

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.

Ultrasonography 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

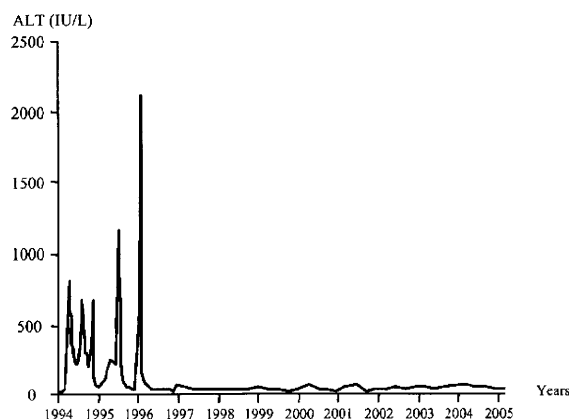


Fig. 3. Average integration value and arithmetic mean value of alanine aminotransferase (ALT) in a 26-year-old patient with hepatitis B virus (HBV). The patient was followed-up for 11.2 years. The number of ALT examinations was 96. The integration value of ALT was 955.2 IU/L \times years. The average 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 $\geq 15.0 \times 10^4 / \text{mm}^3$), 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 $<15.0 \times 10^4/\text{mm}^3$ ($n = 70$) than in patients with platelet counts $\geq 15.0 \times 10^4/\text{mm}^3$ ($n = 311$, $P < 0.001$, 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 $\geq 15.0 \times 10^4/\text{mm}^3$ [$n = 257$]; B: HBV-DNA levels < 5.0 log copies/ml and platelet counts $< 15.0 \times 10^4/\text{mm}^3$ [$n = 45$]; C: HBV-DNA levels ≥ 5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/\text{mm}^3$

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 ($\times 10^4/\text{mm}^3$)	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).

^aPersistently normal ALT values includes patients with ≤ 40 IU/L.

^bCirrhosis 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 ($\times 10^4/\text{mm}^3$)		
≥ 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.

^aPersistently normal ALT values includes patients with ≤ 40 IU/L.

^bCirrhosis diagnosed by ultrasound.

[$n = 54$]; and D: HBV-DNA levels ≥ 5.0 log copies/ml and platelet counts $< 15.0 \times 10^4/\text{mm}^3$ [$n = 25$]). 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

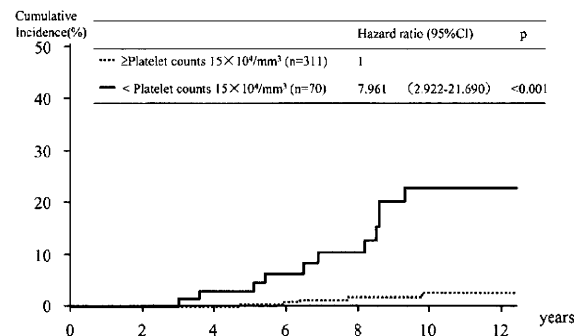


Fig. 4. Incidence of HCC according to platelet counts. The 5- and 10-year cumulative incidences of HCC was 0.4% and 2.6%, respectively, in patients with platelet counts $\geq 15.0 \times 10^4/\text{mm}^3$ ($n = 311$), and 2.9% and 22.9% in patients with platelet counts $< 15.0 \times 10^4/\text{mm}^3$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

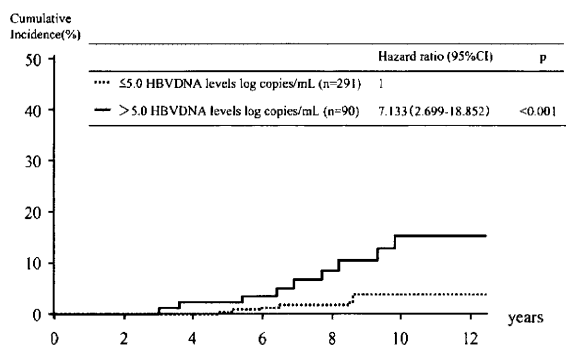


Fig. 5. Incidence of HCC according to serum HBV-DNA levels. The 5- and 10-year cumulative incidences of HCC was 0.4% and 3.7%, respectively, in patients with HBV-DNA levels <5.0 log copies/ml (n=291) and 2.3% and 15.5%, respectively, in patients with HBV-DNA levels ≥5.0 log copies/ml (n=90). The cumulative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

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 >10⁵ 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-Marín et al., 2003]. Thus, it is important for patients to maintain low HBV-DNA levels (i.e., ≤10⁵ 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 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 (×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.

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

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

^aPersistently normal ALT values includes patients with ≤ 40 IU/L.

^bCirrhosis diagnosed by ultrasound findings.

****P* < 0.0001.

*****P* < 0.0005.

was strongly associated with the increased incidence of HCC by univariate analysis. Anatomical constraints and interobserver variability, however, remain limiting factors. In this study, histological confirmation was obtained in only 20 patients (6.3%). It is thought that this study had limitations because the liver histology was not obtained in many cases. Liver biopsy is still the “gold standard” for assessing liver fibrosis; however, it is not practical to undertake biopsies on all patients because of the potential complications which might arise from this procedure. Furthermore, results often differ depending on the pathologist, and results for liver fibrosis in liver biopsy specimens do not always reflect the grade of fibrosis in the entire liver. In contrast, the platelet count is a useful surrogate marker for the

diagnosis of cirrhosis. Lu et al. [2006] reported that the best cutoff platelet count for a diagnosis of cirrhosis is $15.0 \times 10^4/\text{mm}^3$. 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.

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

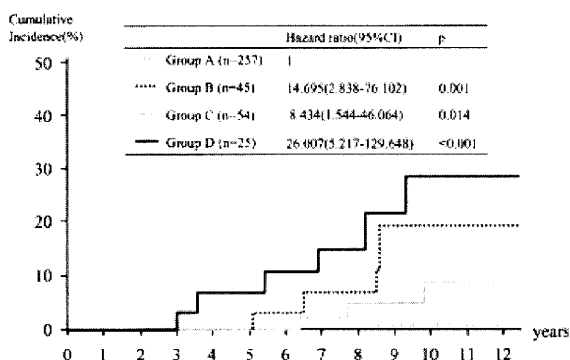


Fig. 6. The cumulative incidence of HCC according to HBV-DNA levels and platelet counts. HBV carriers with normal ALT levels were divided into four groups (A: HBV-DNA levels < 5.0 log copies/ml and platelet counts $\geq 15.0 \times 10^4/\text{mm}^3$ [n = 257]; B: HBV-DNA levels < 5.0 log copies/ml and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

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

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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 non-cancerous 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 develop-

ment 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- μ m 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 centigrade 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

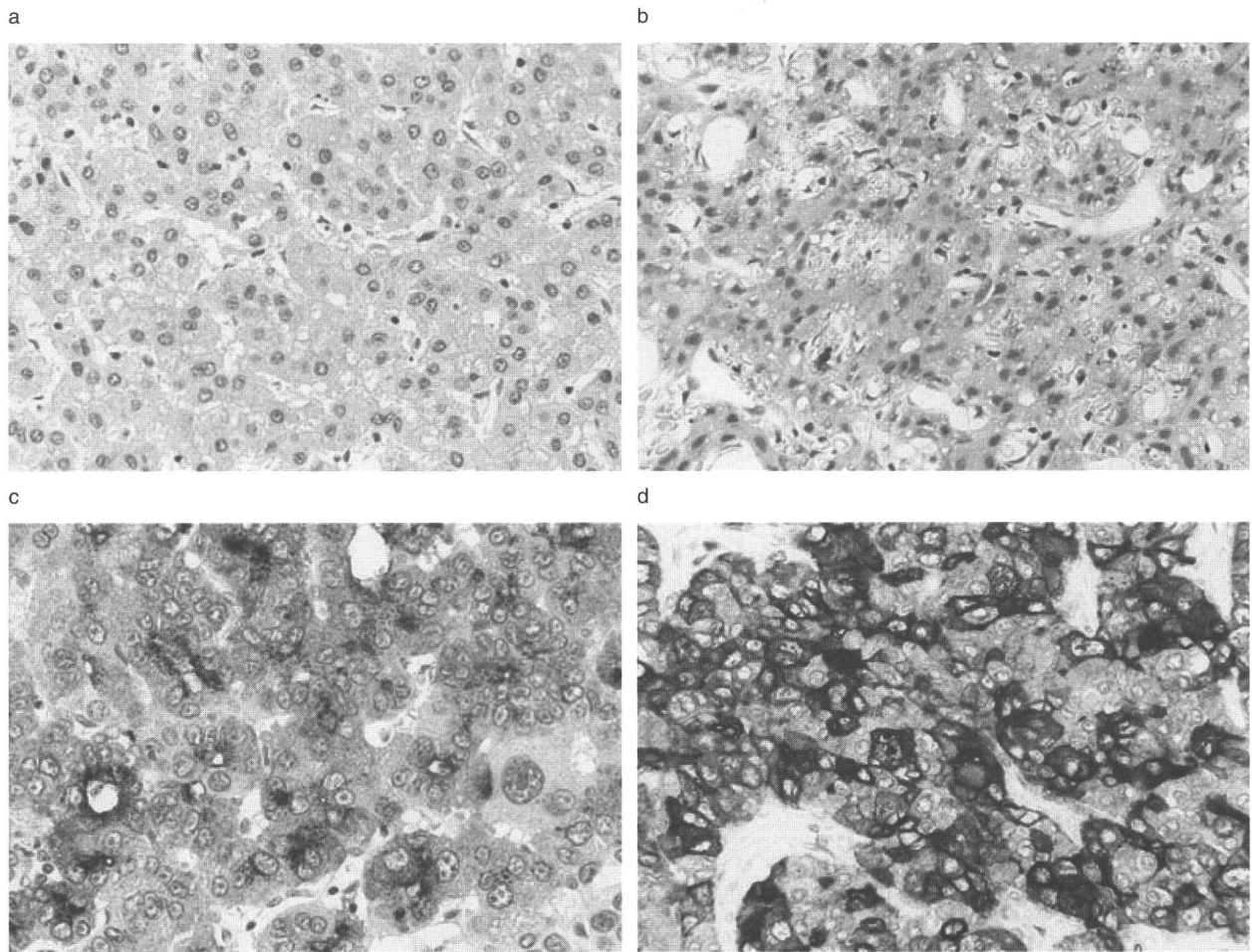


Figure 1 The degree of immunohistochemical staining for glypican-3. (a) No staining, (b) light staining, (c) moderate staining, (d) heavy staining.

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 \pm 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 (<i>n</i> = 200)	Control (<i>n</i> = 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 summarized 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 (<i>n</i> = 200)	Control (<i>n</i> = 200)	<i>P</i> 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% quartile) are shown.

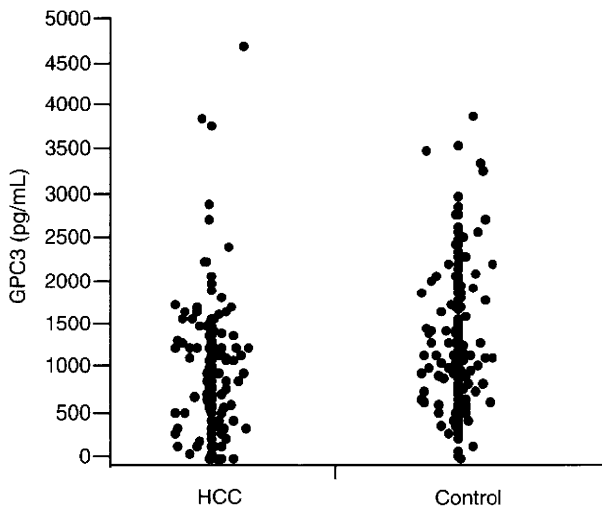


Figure 2 Serum glypican-3 (GPC3) level in patients with hepatocellular carcinoma (HCC) and in patients with chronic liver disease (CLD, control). Serum GPC3 level was higher in patients with CLD (1161.6 pg/mL) than those with HCC (924.8 pg/mL; $P < 0.0001$).

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

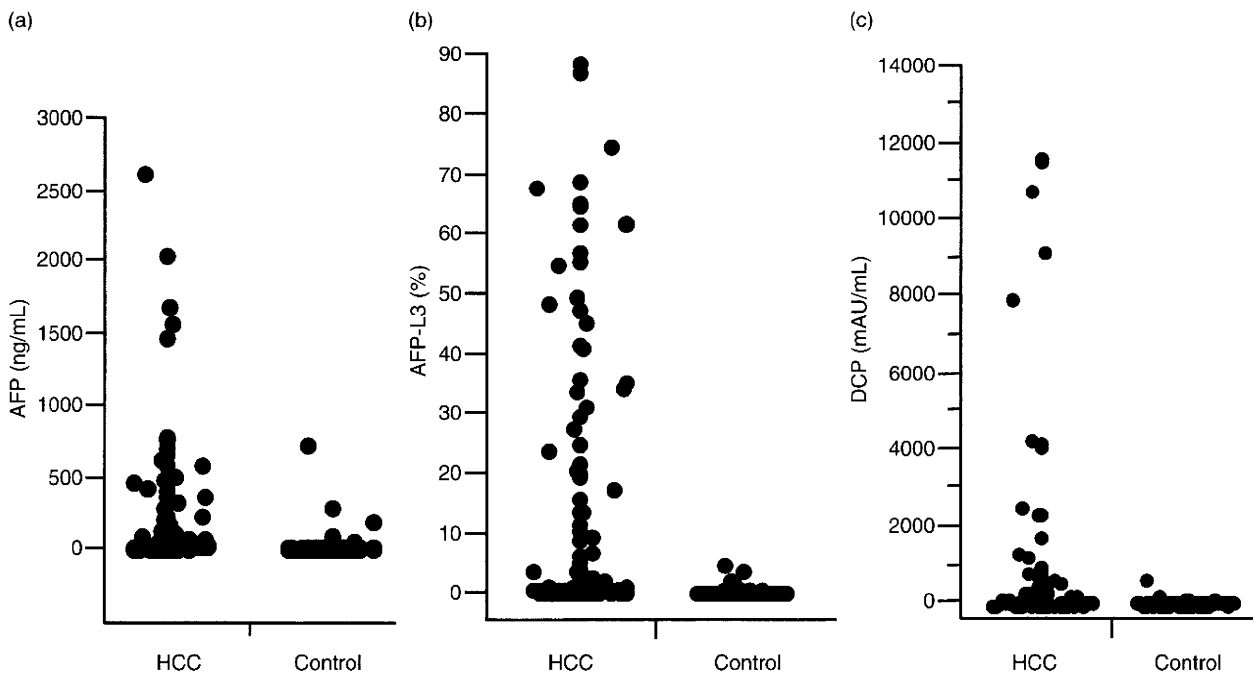


Figure 3 Serum alpha-fetoprotein (AFP), Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and des-gamma carboxy prothrombin (DCP) levels in patients with hepatocellular carcinoma (HCC) and in patients with chronic liver disease (CLD, control). Serum AFP, AFP-L3, and DCP levels were significantly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

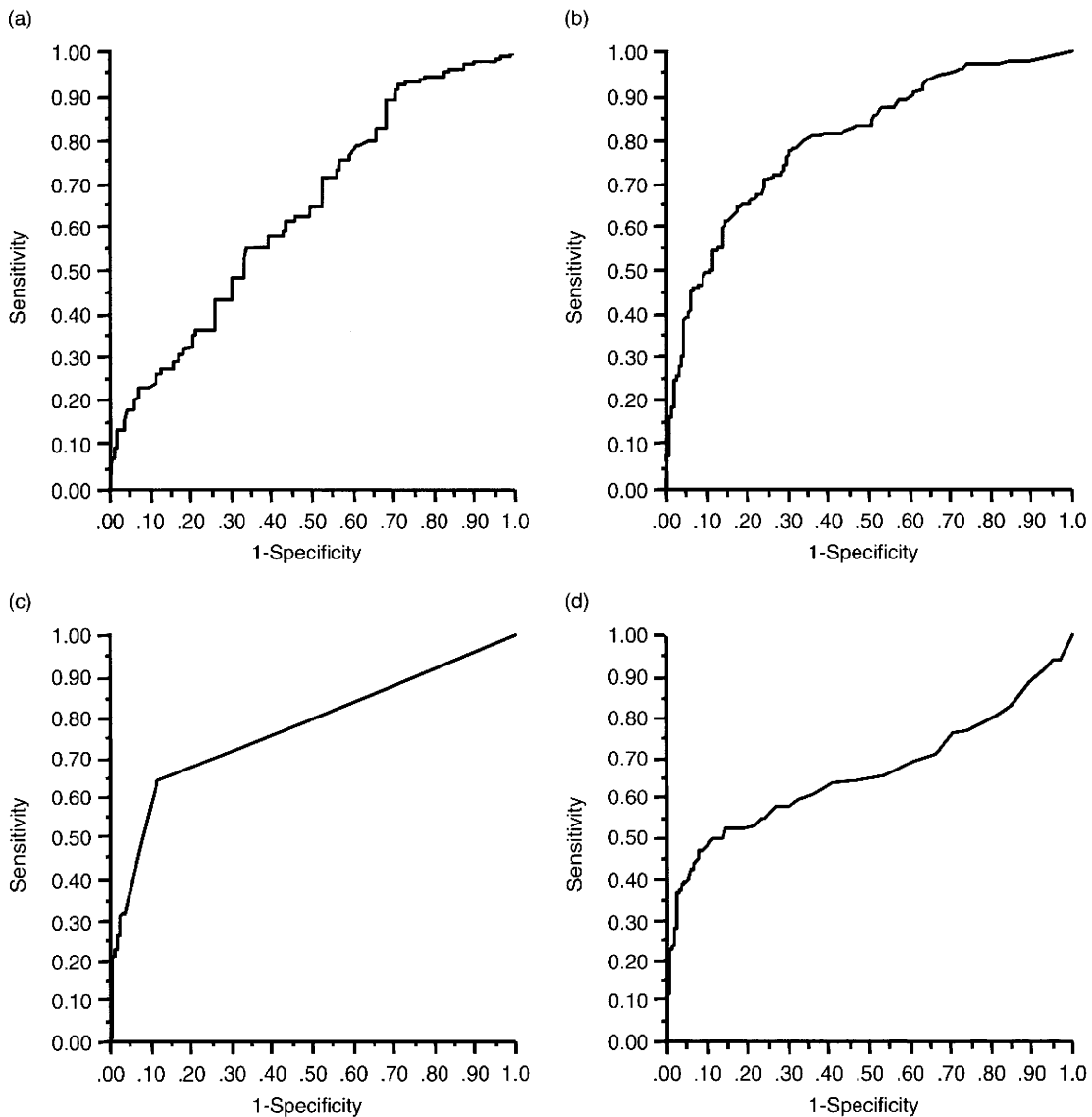


Figure 4 Area under the receiver-operating curve (AUROC) of (a) serum glypican-3 (GPC3), (b) alpha-fetoprotein (AFP), (c) Lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), and (d) des-gamma carboxy prothrombin (DCP) for the diagnosis of hepatocellular carcinoma. AUROC was 0.64 for GPC3, 0.80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

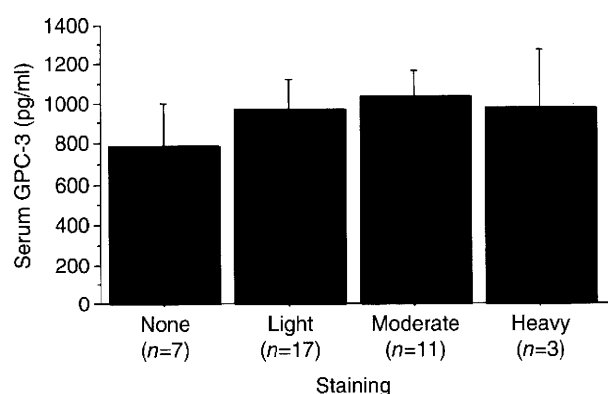


Figure 5 Serum glypican-3 (GPC3) level in 38 patients with hepatocellular carcinoma (HCC) who underwent hepatectomy according to the immunohistochemical staining of GPC3 on the resected HCC specimens. No association was found between serum GPC3 level and immunohistochemical staining of GPC3 on HCC tissues.

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 tissues (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 glycosylphosphatidylinositol 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 NH₂-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 1G12²⁵ 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 GPC3 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 NH₂-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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ペプチドワクチン 国内で臨床試験の行われている ペプチドワクチン療法 GPC3

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Point

- 肝細胞がん特異的ながん胎児性抗原としてGPC3を同定し、GPC3が肝細胞がんの腫瘍マーカーとしても有用であることも示した。
- 進行肝細胞がん患者を対象としたGPC3由来ペプチドワクチンの臨床第I相試験の結果、免疫学的効果や臨床的な効果が認められた。無増悪期間中央値は4ヵ月、全生存期間中央値は9ヵ月であった。
- GPC3ペプチドワクチン3回投与で終了した後は末梢血中ペプチド特異的CTLの頻度は持続せず、4回目以降のワクチンは意味があると考えられた。
- 初回肝細胞がん根治的治療後の再発予防効果を検証する第II相試験を実施中であり、腫瘍量が少なく免疫抑制のない患者での検証が行われている。卵巣明細胞腺がんを対象とした第II相臨床試験も行われている。
- わが国でのがんワクチンの医師主導の臨床試験は、資金面、体制面とも不足しており、国家プロジェクトでしっかりとエビデンスを構築できるような支援が必要とされる。

がんの免疫療法の概念はすでに19世紀からあった。医師たちはがん患者が細菌に感染すると、がんが小さくなる場合があることに気づいていた。そこ

から生まれたColey's vaccine (toxin) は、時にはがんの完全退縮を得たが、広くは受け入れられなかった。また、がんにはまれではあるが、自然退縮が

起こる。これにはおそらく免疫も関与している。1967年ごろには、がん細胞の自家移植の報告がなされている。手術で得られたがん組織からがん細胞をばらばらにして、1万個、10万個、100万個、1,000万個、1億個とその患者の皮下に移植した結果、進行がんの患者でも1万個は完全に拒絶され、10万個では時に移植が成立し、1億個ではほとんど移植が成立することがわかった。これらの研究結果は、がんに対する免疫の確かな存在と、一方ではその限界も示しているといえよう。

1991年にBoonらにより、ヒトの免疫系ががんを異物として認識し、排除しうることによって科学的な根拠が与えられた¹⁾ことによって、またがん免疫研究は勢いを盛り返し現在に至っている。現在までに、さまざまながん拒絶抗原およびペプチドが同定され、世界中で臨床試験が進められている。最近では、前立腺がんに対して「Provenge®」という樹状細胞療法がFDAに承認され話題になったが、その他にもいくつかの第Ⅲ相臨床試験での有効性も報告されている。日本国内でもさまざまな施設からがんに対するペプチドワクチンの有効例の報告が散見される。一方では、最近、子宮頸がんの予防ワクチンが話題であるが、免疫療法がより有効であるのは、がんの再発予防や予防であると考えられ、免疫療法を用いた根治治療後の再発予防法やがん発症予防法の開発も必要である。本稿では、われわれが研究している glypican-3 (GPC3) ペプチドを用いたワクチン療法について述べたいと思う。

がん特異的抗原 glypican-3 (GPC3) の同定

われわれは、東大医科研・ヒトゲノムセンターの中村祐輔教授(現国立がん研究センター研究所長)との共同研究により、cDNA マイクロアレイを利用した2万種類を超える遺伝子の肝細胞がんと正常組織における発現解析データを用いて、肝細胞がん特異的ながん胎児性抗原としてGPC3を同定した。GPC3遺伝子および蛋白質は、ほとんどの肝細胞がん組織ならびに細胞株で高発現するが、正常組織においては、胎生期の肝臓あるいは免疫学的に隔離された胎盤でしか発現がみられない。GPC3は肝細胞がんの腫瘍マーカーとしても有用であることも示した²⁾。われわれは、GPC3が理想的な腫瘍拒絶抗原になりうるかどうかを検討した。日本人の約60%が陽性であるHLA-A24とBALB/cマウスのMHCクラスI分子のK^dにはほぼ同じペプチドが結合することがわかっている。さらに、ヒトとマウスのGPC3では93%のホモロジーを認めることから、HLA-A24、K^dのいずれにも結合しうるGPC3由来のペプチドを合成した。これらをBALB/cマウスに免疫して解析し、K^d拘束性のHLA-A24結合性CTLエピトープペプチド(EYILSLEEL)を同定した³⁾。同様にこれらをHLA-A24 transgenic mouse (Tgm)に免疫して解析した結果もやはり、同じCTLエピトープペプチド(EYILSLEEL)が同定された。このHLA-A24結合性GPC3由来ペプチド(EYILSLEEL)を用いて、

ヒトのHLA-A24陽性の肝細胞がん患者の末梢血リンパ球を刺激することで、約半数からGPC3特異的CTLを誘導することができた⁴⁾。GPC3特異的CTL株を、GPC3高発現ヒト肝細胞がん細胞株を移植したヌードマウスに移入して、その治療効果を証明した⁴⁾。また、日本人の40%が陽性で、欧米白人のメジャータイプであるHLA-A2拘束性のCTLエピトープペプチドを同定するために、HLA-A2に結合しうるペプチドを合成し、これらをHLA-A2 Tgmに免疫して解析した。同定したHLA-A2結合性GPC3由来ペプチド(FVGEFFTDV)を用いてヒトのHLA-A2陽性の肝細胞がん患者の末梢血リンパ球を刺激することで、約半数からGPC3特異的CTLを誘導することができた⁴⁾。また、非常に重要なことであるが、以上のいずれのマウスの実験においても、GPC3抗原の免疫によって、ペプチド特異的CTLが誘導され、抗腫瘍効果は認められたが、自己免疫現象は決して誘導されなかった^{3,4)}。

進行肝細胞がん患者を 対象としたGPC3由来 ペプチドワクチンの臨床 第Ⅰ相試験の結果

平成19年2月、臨床第Ⅰ相試験をスタートし、平成21年11月に完了した。

1回の投与量を0.3、1、3、10、30mgの5段階とし、2週間おきに3回、左右の腋窩部、腹部および鼠径部の皮内に不完全フロイントアジュバント(incomplete Freund's adjuvant; IFA)とともに、安全性を確認しながら

有害事象	Grade 1	Grade 2
アレルギー反応(潮紅あるいは皮疹) 24 (80%)	投与時の一過性の潮紅 22 (73.3%)	異所性の皮疹あるいは潮紅 2 (6.7%)
アレルギー反応(薬剤熱) 6 (20%)	<38℃の薬剤熱 3 (10%)	≥38℃の薬剤熱 3 (10%)
注射部位の反応 30 (100%)	掻痒；紅斑 30 (100%)	炎症反応を伴う疼痛や腫脹 0
掻痒 5 (16.7%)	軽度または限局性の掻痒 5 (16.7%)	激しいまたは広範囲の掻痒 0

表1 CTCAE v3.0による有害事象の評価(計30例中の出現頻度)

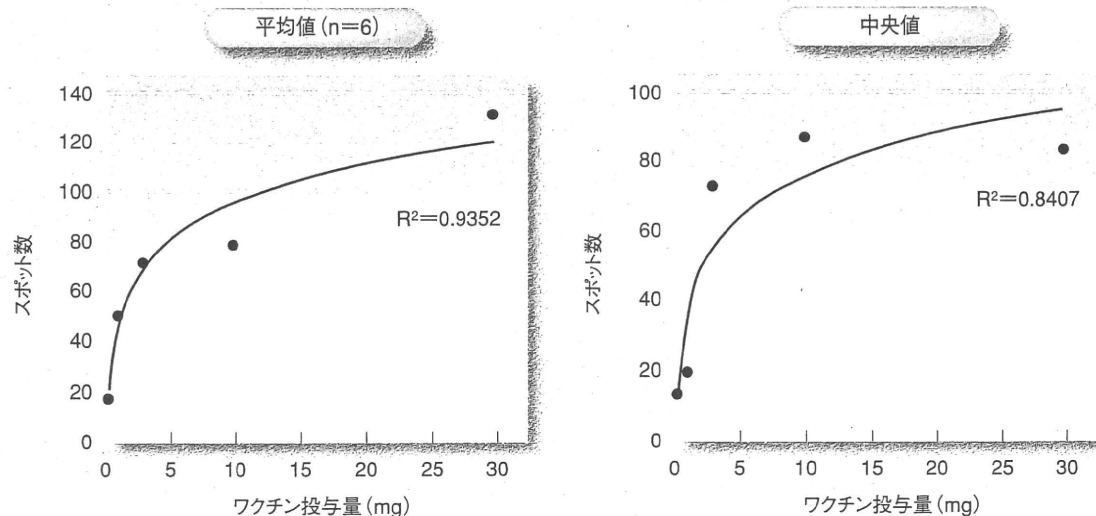


図1 ワクチンの投与量と投与後GPC3特異的CTLの頻度の最大値の相関

用量を増して投与した。

合計30症例全例に用量規制毒性 (dose limiting toxicity ; DLT)は発現せず、安全性に問題はないと判断された。投与時の一過性の潮紅は24例(80%)に認められ、うち1例には一過性の異所性の皮疹、1例には一過性の広範の潮紅を認めた。発熱は37.5℃

以上が6例で、うち38℃以上が3例にみられたがいずれも一過性で、解熱剤の使用を要しなかった。注射部の紅斑は30例全例にみられ、うち5例には軽度の掻痒があり、クロタミトン(オイラックス®)クリームを処方した。炎症反応を伴う疼痛や腫脹は認められなかった(表1)。

免疫学的解析を実施した全30例中27例(90%)に末梢血中ペプチド特異的CTLの頻度の増加が検出され、その頻度は投与量依存性に増加しており(図1)、免疫学的有効性も確認された。7例ではワクチン後の腫瘍の生検を行い、うち5例でワクチン前の腫瘍内には浸潤していなかったCD8陽性

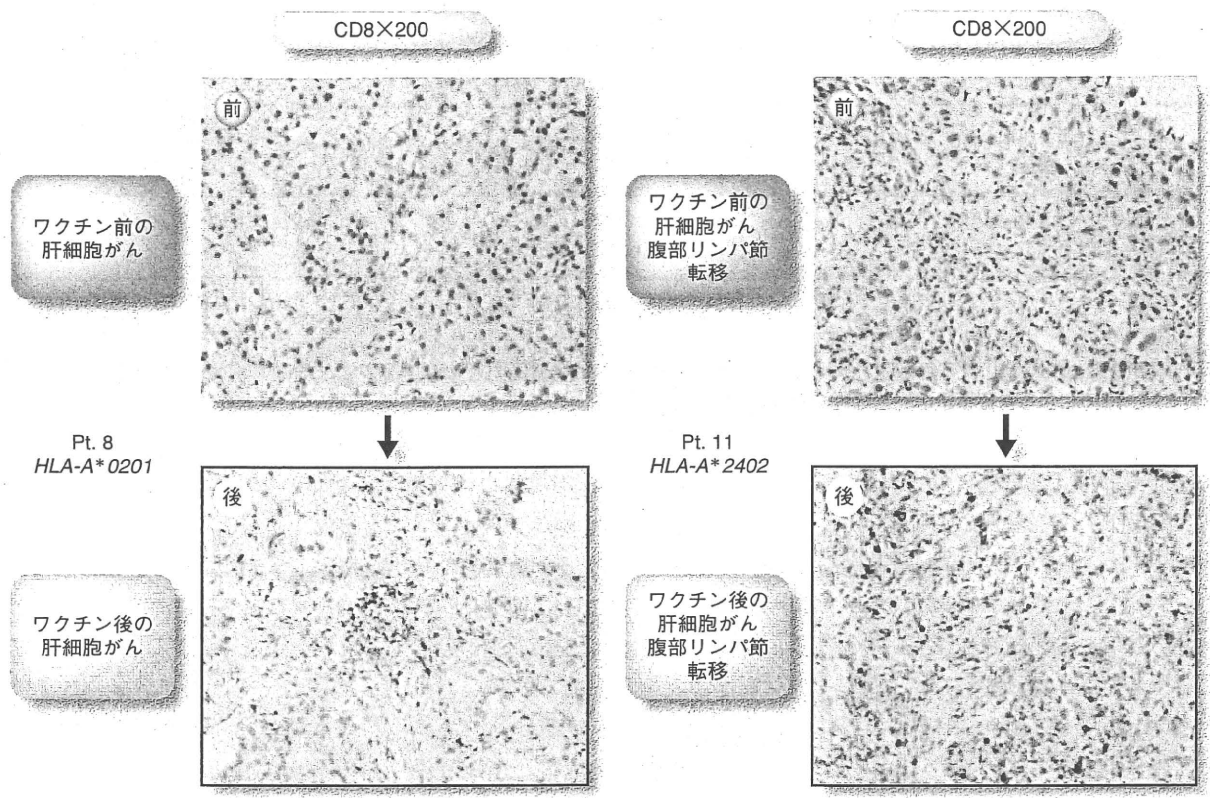


図2 ワクチン前の腫瘍内には浸潤していなかったCD8陽性キラーT細胞がワクチン後の腫瘍内に多数浸潤している2例

のキラーT細胞がワクチン後の腫瘍内に多数浸潤している像も観察できた(図2)。

3回のワクチン投与後1ヵ月後のCTのRECIST version 1.0での評価では、30人中1人がpartial response ; PR(図3)、18人がstable disease ; SD(SD以上63.3%)で、11人はprogression disease ; PDであったが、その内訳は0.3mgの6人中3人(50.0%)、1.0mgでは6人中4人(66.7%)、3.0mgでは6人中4人(66.7%)、10mgでは6人中4人(66.7%)、30mgでは6人中1人の

PRを含む4人(66.7%)がSD以上であり、0.3mgの1人、3.0mgの3人、30mgの1人の計5人には腫瘍内の壊死、一部腫瘍の縮小などの所見も認められた。経過中に一度でもAFP、PIVKA-II、あるいはGPC3の腫瘍マーカーの低下がみられた症例は、29例中22例(75.9%)で、その内訳は、0.3mgの6人中1人(16.7%)、1.0mgでは6人中4人(66.7%)、3.0mgでは5人中5人(100%)、10mgでは6人中6人(100%)、30mgでは6人中6人(100%)と、臨床効果についても投与

量に依存して増加する傾向が示唆された(表2)。

腫瘍量も多く免疫抑制もかかっている患者が多いと考えられる(実際、サイトメガロウイルス由来のペプチドに反応するキラーT細胞の頻度が少ない患者も多かった)今回の進行肝細胞がんの対象に対してわずか3回のワクチン投与で、以上に示したように、免疫学的効果や臨床的な効果が認められた。

全30例の最終結果においても、DLTは1例も発生せず、GPC3ペプチ

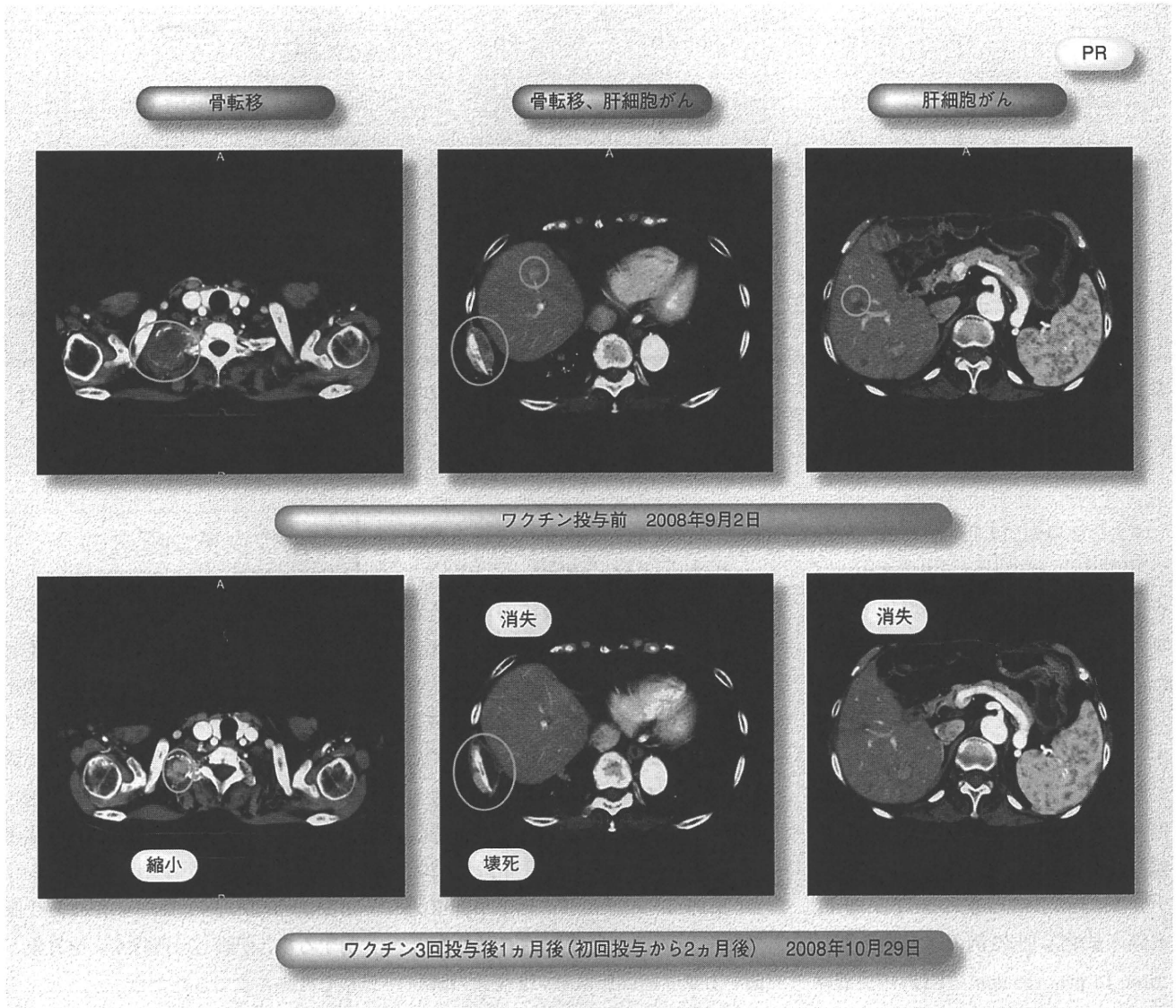


図3 Pt. 24 75歳女性 多発肝細胞がん、骨転移、肺転移、リンパ節転移 HLA-A *0207/1101
HLA-A2 glypican-3 ペプチドワクチン療法：1回30mg、3回投与により著明な臨床効果出現

ドワクチンの安全性は証明されたが、DLTを基に推奨用量を決定することはできなかった。30mg投与の1例にPRの臨床効果が認められたこと、腫瘍マーカーならびに免疫学的モニタリングの結果において用量依存性が認められたことから高用量投与の優位性が

示唆されたものの、30mg投与例では、Grade 2の有害事象が全体の30例中5例(0.3mg投与に1例、3.0mg投与に1例)に対して6例中3例(発熱2例、広範な潮紅1例)と高頻度に認められたこと、3.0mg投与の10倍量の6mLもの量を皮内に投与するため投与手技が

煩雑なうえ、患者の苦痛も大きく、投与部位の発赤・硬結が同じGrade 1でも6例中4例では明らかに大きかったことから、その臨床効果と合わせて考えるとGPC3ペプチドワクチンの推奨投与量は3.0mgが妥当であると判断した。

	PR	SD	PR+SD	PD	腫瘍内の壊死、 一部腫瘍の縮小	腫瘍マーカーの低下 がみられた症例
0.3mg	0	3	3 (50.0%)	3	1	1/6 (16.7%)
1.0mg	0	4	4 (66.7%)	2	0	4/6 (66.7%)
3.0mg	0	4	4 (66.7%)	2	3	5/5 (100%)
10mg	0	4	4 (66.7%)	2	0	6/6 (100%)
30mg	1	3	4 (66.7%)	2	1	6/6 (100%)
計	1	18	19 (63.3%)	11	5	22/29 (75.9%)

表2 3回のワクチン投与後1ヵ月後のCTのRECIST version 1.0での評価と、腫瘍内の壊死、一部腫瘍の縮小がみられた症例数、および、経過中に一度でもAFP、PIVKA-II、あるいはGPC3の腫瘍マーカーの低下がみられた症例数

また、2週間に1回の3回皮内投与で30例中27例において末梢血中ペプチド特異的CTLの頻度が増加する効果は認められたものの、3回投与で終了した後はその頻度は30例中25例で減少し持続しなかった。継続投与を可能とするプロトコル改訂後、12例において継続投与を実施したが、そのうち9例で末梢血中ペプチド特異的CTLの頻度の増加を認め、4回目以降のワクチンは意味があると考えられた。また、本臨床試験参加30例の、無増悪期間 (time to progression ; TTP) 中央値は4ヵ月、全生存期間 (overall Survival : OS) 中央値は9ヵ月であった。

現在実施中もしくは 計画中のGPC3ペプチド ワクチン療法臨床試験

このようなワクチン療法は元来、腫瘍がない、あるいはCTで見えない腫瘍があったとしても腫瘍量が少ない状態でこそ威力を発揮すると考えら

れ、現在、手術やラジオ波焼灼療法 (radiofrequency ablation ; RFA) による初回肝細胞がん根治的治療後の再発予防効果を検証する第II相試験を実施中である。進行肝細胞がん患者にとって有用であるかを検証する第II相試験としては、ソラフェニブとGPC3ペプチドワクチンの併用療法とソラフェニブを比較する非盲検多施設共同ランダム化比較第II相臨床試験と、GPC3ペプチドワクチン単独療法の腫瘍局所での免疫学的有効性を評価する第II相臨床試験の2種類の試験を計画している。

GPC3は肝細胞がんだけでなく、一部の小児がん(肝芽腫、神経芽腫、腎芽腫)、卵巣明細胞腺がん、肺扁平上皮がんにも発現しており、それらのがん種に対する応用も期待される。卵巣明細胞腺がんを対象とした第II相臨床試験はすでに名古屋大学で開始されている。GPC3を発現する小児がんおよび肺扁平上皮がんを対象とした臨床試験も計画している。

がんワクチン療法の 臨床導入への問題点

まず、免疫療法自体が、そもそも有望な治療法と思われてこなかったことがブレーキをかけてきた。ペプチドワクチンは初めて科学的根拠をもって開発された免疫療法であったが、当初のペプチドワクチンの臨床第I相の臨床試験は従来の抗がん剤と同じくもう治療法のない、そもそも有効性を証明することが難しい進行がんを対象として実施されてきたため、かえって失望感を与えてしまった。米国NCIのRosenberg SAらが、2004年のNat Med.にがんワクチンのReviewとして、440例中complete response ; CR、PRのresponse rateはわずか2.6%であったと報告した⁵⁾ことがさらに追い討ちをかけた。この点に関しては、われわれも含めた日本各地のグループから有効例の報告も増えてきて、エビデンスも次第に証明されつつあり、その結果、国内の製薬企業の参画も出