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Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker☆

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Abstract

With the global pandemic of hepatitis B and C infections, the incidence of Hepatocellular carcinoma (HCC) is rapidly increasing world wide. We identified glypican-3 (GPC3), a novel oncofetal gene over-expressed specifically in human HCC, as based on data of cDNA microarrays. As GPC3 is a GPI-anchored membrane protein and could be secreted, we attempted to detect secreted GPC3 protein in sera from HCC patients using Western blotting and ELISA. GPC3 protein was positive in sera of 40.0% (16/40) of HCC patients, and negative in sera from subjects with liver cirrhosis (LC) (0/13), chronic hepatitis (CH) (0/34), and healthy donors (0/60). All subjects were Japanese. Although 12 of 40 HCC patients were negative for both α-fetoprotein (AFP) and PIVKA-II well known tumor markers of HCC, four of these were GPC3-positive in the sera. We also observed vanishing GPC3 protein in the sera of three patients after the surgical treatment for HCC. On the other hand, immunohistochemical analysis revealed that HCC expressed GPC3 protein in all 14 HCC patients tested. In conclusion, GPC3, as defined in this study was shown to be a useful tumor marker for cancer-diagnosis for large numbers of patients with HCC.

Keywords: Glypican-3; Hepatocellular carcinoma; Tumor marker; cDNA microarray; Oncofetal protein

Primary hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. Because of the global pandemic of hepatitis B and C viral infections, the incidence of HCC is rapidly increasing in Asian and Western countries [1,2], and this trend is expected to continue for the next 50 years because of the long latency between infection and the development of HCC. The prognosis of advanced HCC remains poor, and novel treatment and diagnosis strategies are urgently needed.

cDNA microarray technology, by which investigators can obtain comprehensive data with respect to gene-expression profiles, is rapidly progressing. Several studies have demonstrated the usefulness of this technique for identification of novel cancer-associated genes and for classification of human cancers at the molecular level [3]. We identified genes of which expression was altered during hepatocarcinogenesis in 20 subjects with primary HCCs through the use of a genome-wide cDNA microarray containing 23,040 genes [4].

In the present work, we identified glypican-3 (GPC3) over-expressed specifically in human hepatocellular carcinoma, as based on cDNA microarray data. We

☆ *Abbreviations:* HCC, hepatocellular carcinoma; GPC3, glypican-3; HD, healthy donor; LC, liver cirrhosis; CH, chronic hepatitis; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II.

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detected soluble GPC3 protein in sera of HCC patients, but not in case of other liver diseases or other cancers. We propose that GPC3 is a novel tumor marker for HCC.

Materials and methods

cDNA microarrays. Profiling of gene expression by cDNA microarrays was done, as reported [4]. Primary HCCs and corresponding non-cancerous liver tissues were obtained with informed consent from 20 Japanese patients who underwent hepatectomy in the Department of Gastroenterological Surgery, Kyoto University Graduate School of Medicine. These specimens were used only for cDNA microarray analysis, as reported [4]. Poly(A)$^+$ RNAs isolated from human bone marrow, brain, heart, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, stomach, testis, thymus, thyroid, uterus, fetal brain, fetal kidney, fetal liver, fetal lung (Clontech), colon, and ovary (Biochain) were used as probes for the cDNA microarray [5].

HCC tissues, blood samples, and cell lines. After obtaining informed written consent, we independently obtained tissue and blood samples at random from HCC patients and from various donors treated in the Department of Surgery II, and the Third Department of Internal Medicine, Kumamoto University School of Medicine.

We collected patient profiles from medical records to determine the clinical stages, according to the UICC TNM classification (Table 1). Hep G2, Hep 3B, PLC/PRF/5, and HuH-7 were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University, and SK-Hep-1 was provided by Dr. K. Itoh of Kurume University, Kurume, Japan. Hep G2, Hep 3B, PLC/PRF/5, and HuH-7 were cultured in DMEM supplemented with 10% FCS, and SK-Hep-1 was cultured in RPMI1640 supplemented with 10% FCS.

RT-PCR. RT-PCR was done, as described [6]. We designed GPC3 gene-specific PCR primers to amplify fragments of 939 bp and used RT-PCRs consisting of initial denaturation at 94 °C for 5 min and 30 amplification cycles at an annealing temperature of 58 °C. GPC3 PCR primer sequences were: sense, 5′-GTTACTGCAATGTGGTCATGC-3′ and antisense, 5′-CTGGTGCCCAGCACATGT-3′. β-Actin: sense, 5′-CCTCGCCTTTGCCGATCC-3′ and antisense, 5′-GGATCTTC ATGAGGTAGTCAGTC-3′. After normalization by β-actin mRNA, as a control, we compared the expression of GPC3 mRNA in HCC tissues and cell lines.

Western immunoblot analysis. Cell samples were lysed in appropriate amounts of lysing buffer 150 mM NaCl, 50 mM Tris, pH 7.4, 1% Nonidet P-40, 1 mM sodium orthovanadate (Wako, Osaka, Japan), 1 mM EDTA, plus a protease inhibitor tablet (Amersham, Arlington Heights, IL). Supernatant fluids of the lysates were electrophoresed on SDS–PAGE gels and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking with 5% skimmed milk and 0.2% Tween 20 in Tris-buffered saline, the membrane was incubated with the anti-GPC3, rabbit polyclonal antibody raised against a recombinant protein corresponding to human GPC3 303–464 (Santa Cruz, California), washed extensively with PBS, and subjected to chemiluminescence detection using peroxidase-conjugated anti-rabbit Ig, horseradish peroxidase linked F(ab′)$_2$ fragment (from donkey) (Amersham), using an ECL kit (Amersham). We purchased GPC3 303–464 produced in *Escherichia coli* as a 45 kDa tagged fusion protein (Santa Cruz, CA) and this protein was added in SDS–PAGE loading buffer to serve as a positive control.

Immunohistochemical examination and ELISA. Immunohistochemical examinations were done, as described [5]. We stained 4-μm-thick sections of formalin-fixed and paraffin-embedded tissue samples with anti-GPC3 Ab at a dilution of 1:200. To set up ELISA detection of GPC3 in sera from patients and healthy donors, we purchased FluoReporter Mini-Biotin-XX Protein Labeling Kits (F-6347) (Molecular Probes, Eugene) for biotinylating anti-glypican-3, rabbit polyclonal antibody. The 96-well ELISA plates (Nunc, Denmark) were coated overnight at 4 °C with 0.1 μg/well anti-human GPC3 303–464 (Santa Cruz) in PBS, pH 7.4. Then, the plates were blocked with Block Ace (Dainippon pharmaceutical, Osaka) for 1 h at room temperature. Serum samples from patients and healthy donors were diluted at 1:200 with 10× Block Ace to serve as samples for ELISA. We added standard samples of positive control and culture supernatants with biotinylated anti-GPC3 Ab, followed by incubation for 2 h at room temperature. After washing three times with PBS, HRP-Conjugated Streptavidin (ENDOGEN, Woburn) was added to each well. After 30 min of incubation, the plates were washed three times with PBS and TMB Substrate Solution (ENDOGEN) was added. We then used an

Table 1
Profiles of serum donors and detection of GPC3 using ELISA

Disease	Mean age (years)	Sex		Virus-positive donor[a]			UICC stage[b]				GPC3 positive rate
		M	F	HCV	HBV	Non-B–non-C	I	II	III	IV	
HCC	66	36	4	27	8	6	1	15	14	10	16/40 (40%)
Liver cirrhosis	65	6	7	8	4	1					0/13 (0%)
Chronic hepatitis	60	15	19	31	3	0					0/34 (0%)
Autoimmune hepatitis	65	0	2								0/2 (0%)
Primary biliary cirrhosis	79	0	1								0/1 (0%)
Healthy donors	40	25	35								0/60 (0%)
Cancers											
Colon	66	16	5				1	6	5	9	0/21 (0%)
Gastric	71	9	5				7	3	4	0	0/14 (0%)
Pancreatic	58	6	5				0	0	0	11	0/11 (0%)
Biliary	70	2	4				0	3	1	2	0/6 (0%)
Esophageal	59	6	0				1	0	2	3	0/6 (0%)
Lung	64	7	0				3	0	0	4	0/7 (0%)
Breast	50	0	10				4	2	2	2	0/10 (0%)

[a] HCV was detected using RT-PCR. HBsAg was examined using radioimmunoassays.
[b] International Union Against Cancer Classification; TNM Classification of malignant tumors.

ELISA reader (model 550, Bio-Rad) at 450 nm for measurement of optical density (OD).

Results

Identification of the GPC3 gene over-expressed specifically in HCC

We obtained data comparing expression profiles between 20 HCCs (10 cases were hepatitis B virus (HBV)-positive and 10 were hepatitis C virus (HCV)-positive) and their corresponding non-cancerous liver tissues [4] and in various normal human tissues [6], using cDNA microarrays. We then searched for genes over-expressed specifically in HCC using these data and we identified GPC3. In 16 cases of the 20 HCCs, the expression of GPC3 mRNA in the cancer tissue was 5 or more times higher than that in non-cancerous tissues (Fig. 1A). GPC3 is an over-expressed gene in most HCCs and is not related to HBV or to HCV viral infection. GPC3 mRNA is highly expressed in the placenta, fetal liver, fetal lung, and fetal kidney and is low in most adult normal tissues (Fig. 1A). Data on GPC3 have been published by other investigators, as based on Northern blotting studies [7,8]. Thus, like AFP, GPC3 is a novel onco-fetal antigen in HCC.

Expression of GPC3 mRNA in human HCC

We examined GPC3 mRNA expression using RT-PCR. HCC tumor of 6 patients, 37, 35, 34, 38, 42, and 43 among 7 patients tested (85.7%), showed a much stronger expression than did non-cancerous liver tissue in these patients. The HCC tumor of patient 41 showed no such expression (Fig. 1B). Hep G2, Hep 3B, and HuH-7 HCC cell lines showed a stronger expression of GPC3 mRNA than PLC/PRF/5 while, SK-Hep-1 showed no such expression (Fig. 1C).

The presence of soluble GPC3 protein in culture supernatants of HCC cell lines and sera from HCC patients

As GPC3 is a GPI-anchored membrane protein and could be secreted, we next attempted to detect secreted GPC3 protein. We used Western blotting techniques for Hep G2 cell lysates and the culture supernatant harvested after the indicated culture period to gain support for the existence of soluble GPC3 protein in culture supernatants of Hep G2. Hep G2 cell lysates prepared from 1×10^5 cells after cultivation for 6, 12, 24, and 48 h (lanes 1, 3, 5, and 7 in Fig. 2A) in serum-free medium showed similar amounts of 60 kDa GPC3 protein. On the other hand, Hep G2 culture supernatants (20 μl of 1 ml/well) after cultivation for 6, 12, 24, and 48 h (lane 2, 4, 6, and 8) showed a gradual increase in 60 kDa GPC3

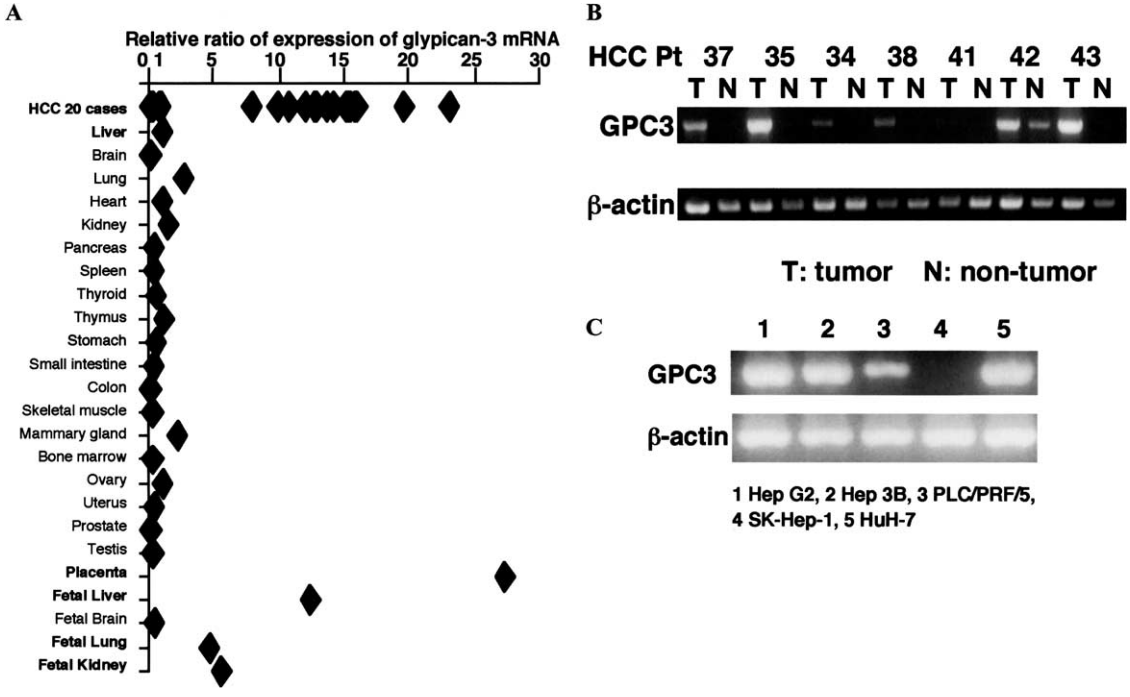

Fig. 1. HCC-specific expression of GPC3 mRNA. (A) The relative ratio (RR) of expression of human GPC3 mRNA in 20 HCC patients and in disease-free tissues. RR in HCC show cancerous tissue versus adjacent non-cancerous liver tissue intensity ratio in each case. RR in disease-free tissues show each disease-free tissue versus disease-free liver intensity ratio. (B) Expression of GPC3 mRNA detected using RT-PCR in human HCC tissues. (C) Expression of GPC3 mRNA detected using RT-PCR in human HCC cell lines.

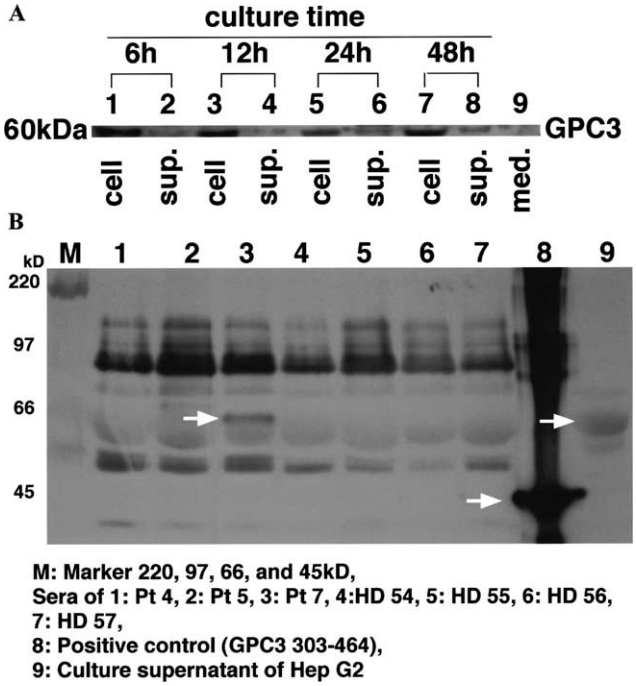

Fig. 2. Evidence for secretion of GPC3 from human HCC. (A) Evidence for the presence of GPC3 protein in the culture supernatant of Hep G2 as based on Western blots. Lanes 1, 3, 5, and 7: lysate of 1×10^5 Hep G2 cells after cultivation for 6, 12, 24, and 48 h. Lanes 2, 4, 6, and 8: 20 μl of Hep G2 culture supernatant after cultivation for 6, 12, 24, and 48 h. (B) Evidence for soluble GPC3 protein in sera from HCC patients. Arrows indicate bands of GPC3 protein. Lane 8: positive control, 45 kDa GPC3 303–464. Lane 3: 20 μl of sera of Pt. 7. Lane 9: culture supernatant of Hep G2.

protein, that is, GPC3 protein was indeed secreted from Hep G2 into the culture supernatant.

We then searched for soluble GPC3 protein by Western blotting of sera of three HCC patients and four healthy donors (HDs). We detected the band of positive control, 45 kDa GPC3 303–464 (lane 8 in Fig. 2B), and detected the band of 60 kDa GPC3 protein in 20 μl of sera from Pt 7 (lane 3 in Fig. 2B) and in culture supernatant of Hep G2 (lane 9 in Fig. 2B), but not in sera from two other HCC patients (patients 4 and 5) or from four healthy donors (HDs 54–57).

We next detected soluble GPC3 using ELISA. We defined the concentration of GPC3 protein in the 1ml of the culture supernatant of 1×10^5 Hep G2 cells after cultivation for 24 h as 1 U/ml. The amount of GPC3 protein in the culture supernatant of the Hep G2, PLC/PRF/5, and HuH-7 was much larger than that of the Hep 3B, and that of the SK-Hep-1 was not detected (Fig. 3A), although the amount of GPC3 mRNA of the Hep 3B was much larger than that of the PLC/PRF/5 (Fig. 1C). Thus, there was some discrepancy between the expression levels of GPC3 mRNA in HCC cells and the amount of GPC3 protein secreted into the culture supernatant.

The quantification by ELISA of GPC3 protein in sera of 40 HCC, 13 LC, 34 CH, and other patients and of 60 HDs is indicated in Figs. 3B and C, Tables 1 and 2. As we did not have recombinant GPC3 protein useful for a positive control for this ELISA system, serially diluted culture supernatant of Hep G2 was used to estimate the

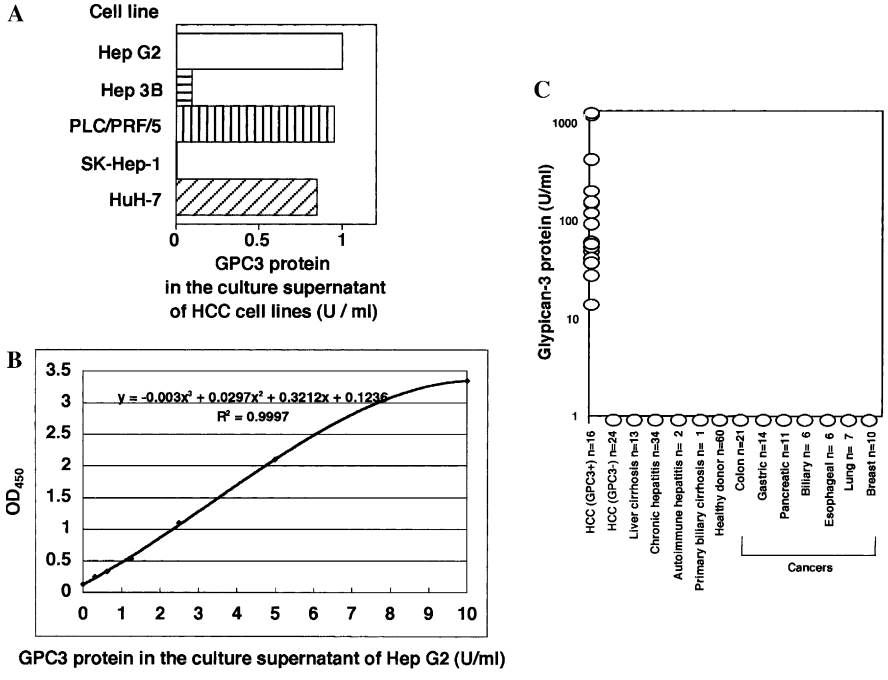

Fig. 3. Quantification of GPC3 protein using ELISA. (A) Quantification of GPC3 protein secreted in the culture supernatant of HCC cell lines. We defined the concentration of GPC3 protein in 1 ml of the culture supernatant of 1×10^5 Hep G2 cells after cultivation for 24 h to be 1 U/ml. (B) Standard curve to quantify the GPC3 protein based on OD data. Serially diluted culture supernatant of Hep G2 was used to estimate the standard curve. (C) Quantification of GPC3 protein in sera from 40 HCC patients and other patients with liver diseases or other cancers and healthy donors.

Table 2
Profiles of 40 Japanese patients with HCC and quantification of AFP, PIVKA-II, and GPC3 in sera of these patients

Pt ID	Age (years), sex	Virus[a]	UICC stage	AFP (ng/ml)[b] (<20)[c]	PIVKA-II (mAU/ml)[d] (<40)	GPC3 (U/ml) (<10)
3	56, M	HBV	IVA	54[e]	957	51
5	71, M	HCV	IIIB	8900	31,577	15
11	69, M	Non-B, non-C	IVA	9400	319	215
32	71, M	Non-B, non-C	II	30	508	30
1	64, M	HCV	IIIA	50	15	448
2	53, M	HCV	II	45	38	162
8	69, M	HCV	III	21	21	99
39	78, M	HCV	IIIA	94	25	45
9	73, M	HCV	IIIA	25	242	–
12	61, M	HCV	IVA	349	169	–
17	70, M	HBV	IVA	56	133	–
18	71, F	HBV	II	930	994	–
19	77, M	HCV	II	163	96	–
24	50, M	HCV	II	29	41	–
26	63, M	HBV	IVA	5280	1549	–
38	60, M	HCV	II	16,200	3556	–
4	69, M	HCV	IIIA	178	28	–
15	67, M	HCV	II	100	32	–
22	72, M	HCV	IIIA	25	12	–
33	60, M	HCV	IIIA	2030	14	–
7	62, M	HCV	IVA	10	239	1301
16	72, M	HCV	II	<1	840	130
30	75, M	HCV	II	5	102	166
37	59, M	Non-B, non-C	II	3	707	65
14	62, M	HCV	II	3	69	–
20	75, F	Non-B, non-C	IIIA	<1	63	–
27	71, M	HCV	IVA	10	19,288	–
35	52, M	HBV	II	13	431	–
6	72, F	HCV	II	9	20	58
25	63, M	HCV	IVB	<1	<10	1349
29	56, M	Non-B, non-C	II	<1	22	62
40	58, M	HBV	II	3	11	40
10	58, M	Non-B, non-C	IIIA	3	18	–
13	69, M	HCV	IVB	6	<10	–
21	71, M	HBV, HCV	IVA	2	26	–
23	59, M	HBV	IIIA	3	18	–
28	69, M	HCV	IIIA	<1	28	–
31	69, M	HCV	IIIA	17	13	–
34	63, F	HCV	I	4	14	–
36	61, M	HCV	IIIB	13	29	–

[a] HCV was detected using RT-PCR. HBsAg was examined using radioimmunoassay.
[b] AFP was quantified using radioimmunoassay.
[c] Values in parentheses represent cut-off value.
[d] PIVKA-II was quantified using enzyme immunoassays.
[e] Positive values are underlined.

standard curve to quantify the GPC3 protein based on OD data (Fig. 3B). We detected and quantified GPC3 protein in the sera of 16 of 40 HCC patients, but not in sera of patients with liver cirrhosis (LC), chronic hepatitis (CH), autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), HD, and another kinds of cancers. Fig. 3B shows the standard curve for ELISA-detection of GPC3 that guarantees the quality of this ELISA. According to these data, we were convinced that the lowest limit for detection of serum GPC3 was 10 U/ml by using 200× diluted serum samples and we determined more than 10 U/ml to be positive. The evidence that our ELISA system detected soluble GPC3 in culture supernatant of NIH3T3 transfected with mouse GPC3 gene but not in that of wild type NIH3T3 cells also supports the accuracy of ELISA (data not shown).

The prevalence of GPC3 protein in the sera of HCC patients was significantly higher than that in other donors ($P < 0.0001$). The GPC3 evaluated by ELISA for patients 4, 5, and 7 is given in Fig. 2B and was 0, 15, and 1301 U/ml, respectively (Table 2). Namely, 1301 U/ml of GPC3 protein was detectable and 15 U/ml of GPC3 was not detectable using Western blotting techniques. Although as shown in Fig. 1B, HCC cells of Pt. 35 showed much stronger expression of GPC3 mRNA than did those of Pts. 37, 34, and 38, serum GPC3 was detected

Table 3
Lack of correlation between serum GPC3 levels and injury of hepatocytes or liver function in 40 Japanese patients with HCC

Pt ID	GPC3 (U/ml)	Injury of hepatocytes		Liver function				
		AST[a] (IU/l) (<40)[b]	ALT[c] (IU/l) (<40)	Ascites	T-Bil[d] (mg/dl) (<1.0)	Alb[e] (g/dl) (>4.0)	$ICGR_{15}$ (%)[f] (<15)	PT[g] (%) (>70)
25	1349[h]	38	36	−	1.0	3.7	37.0	68
7	1301	104	53	+	1.2	3.7	43.0	65
1	448	138	166	−	1.0	3.5	31.3	90
11	215	22	14	−	0.6	3.7	12.7	97
30	166	60	59	−	0.7	3.6	15.1	85
2	162	47	68	−	1.5	4.6	6.8	82
16	130	18	9	−	0.4	3.8	9.8	91
8	99	96	59	−	1.0	4.2	23.9	99
37	65	26	34	−	0.6	4.3	9.2	94
29	62	23	9	−	0.8	4.6	8.6	110
6	58	55	60	−	1.1	3.7	18.4	92
3	51	341	157	−	1.1	4.0	33.9	96
39	45	63	50	−	1.4	3.3	45.4	82
40	40	23	25	−	0.7	4.1	11.8	91
32	30	44	47	+	1.1	3.8	20.4	83
5	15	65	46	−	0.9	3.2	22.5	81
	Positive rate	10/16 (62.5%)	10/16 (62.5%)	2/16 (12.5%)	6/16 (37.5%)	10/16 (62.5%)	10/16 (62.5%)	2/16 (12.5%)
4	—	36	20	−	1.2	3.0	40.6	77
9	—	101	70	−	0.8	2.6	34.3	85
10	—	37	57	−	1.0	4.2	12.9	104
12	—	62	49	−	1.1	3.7	28.7	77
13	—	163	72	−	0.4	2.1	33.6	90
14	—	55	21	−	1.2	3.3	50.5	69
15	—	39	37	−	1.3	3.1	39.0	59
17	—	62	74	−	0.6	3.7	24.9	77
18	—	29	22	−	0.6	3.6	27.9	86
19	—	23	10	−	1.6	2.8	13.9	66
20	—	55	29	+	1.1	3.5	47.0	66
21	—	47	37	−	1.0	3.2	36.4	85
22	—	78	56	+	1.0	2.9	54.0	77
23	—	37	57	−	1.0	4.2	12.9	104
24	—	49	41	−	0.8	3.9	16.0	97
26	—	19	20	−	0.7	3.7	12.4	87
27	—	38	36	−	1.2	3.0	48.1	84
28	—	54	52	−	1.0	3.7	14.5	85
31	—	56	50	−	1.5	3.1	21.7	85
33	—	52	55	+	1.1	3.0	41.4	77
34	—	29	32	−	1.6	4.6	10.9	90
35	—	32	51	−	0.5	4.1	14.5	89
36	—	67	70	−	0.4	4.0	22.0	87
38	—	64	89	−	1.0	4.3	17.2	96
	Positive rate	14/24 (58.3%)	14/24 (58.3%)	3/24 (12.5%)	10/24 (41.7%)	17/24 (70.8%)	17/24 (70.8%)	4/24 (16.7%)

[a] Serum levels of aspartate transaminase.
[b] Values in parentheses represent cut-off value.
[c] Serum levels of alanine transaminase.
[d] Serum levels of total bilirubin.
[e] Serum levels of albumin.
[f] Indocyanine green retention at 15 min level.
[g] Prothrombin test.
[h] Positive values are underlined.

only in Pt. 37 suggesting that there was no correlation between serum GPC3 concentrations and mRNA expressions of GPC3 in the cancer tissues. We propose that GPC3 may be a novel tumor marker for HCC. There was no correlation in the positive state of tumor markers among the three markers, α-fetoprotein (AFP), protein induced by vitamin K absence or antagonist-II (PIVKA-II), and GPC3 (Table 2). Although 12 patients were negative for both AFP and PIVKA-II, four patients (6, 25, 29, and 40) of 12 were GPC3-positive, and HCC Pts 6, 29, and 40 were classified as a relatively early UICC Stage II (Table 2).

Lack of correlation between serum concentrations of GPC3 and the activity of hepatitis or liver function

As shown in Fig. 1A, GPC3 mRNA expression was found in fetal liver as well as in placenta, suggesting that regenerating hepatocytes in injured liver may express GPC3. To investigate this possibility, relationship between serum concentrations of GPC3 and injury to hepatocytes or liver function were assessed in patients with HCC (Table 3). Serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) are good indexes for injury to hepatocytes. The presence of ascites, serum levels of total bilirubin (T-Bil) and albumin (Alb), indocyanine green retention at 15 min level ($ICGR_{15}$ (%)), and prothrombin test (PT (%)) were chosen as indexes of liver function. There was no significant difference in positive rates of abnormalities in these factors between 16 serum GPC3-positive patients and 24 negative patients. Furthermore, GPC3 was not detected in the sera of any patient with active CH, and serum concentrations of GPC3 were not increased when regeneration of liver occurred after surgical resection of HCC. These data clearly show no correlation between serum concentrations of GPC3 and the activity of hepatitis or liver function.

GPC3 protein in the sera of HCC patients disappeared after surgical treatments

Changes in serum levels of three tumor markers, AFP, PIVKA-II, and GPC3, and tumor masses detected by computed tomography (CT) before and after surgical treatments for HCC in three patients (Pts. 30, 40, and 37) are shown in Fig. 4. GPC3 protein was detectable in these three patients prior to surgery, but GPC3 was not detectable after the surgical treatments for patients with HCC. It should be noted that GPC3 was the only useful tumor marker for Pt. 40.

Expression of GPC3 protein in human HCC tissues

We made an immunohistochemical analysis of GPC3 in HCC and non-cancerous liver tissue surrounding

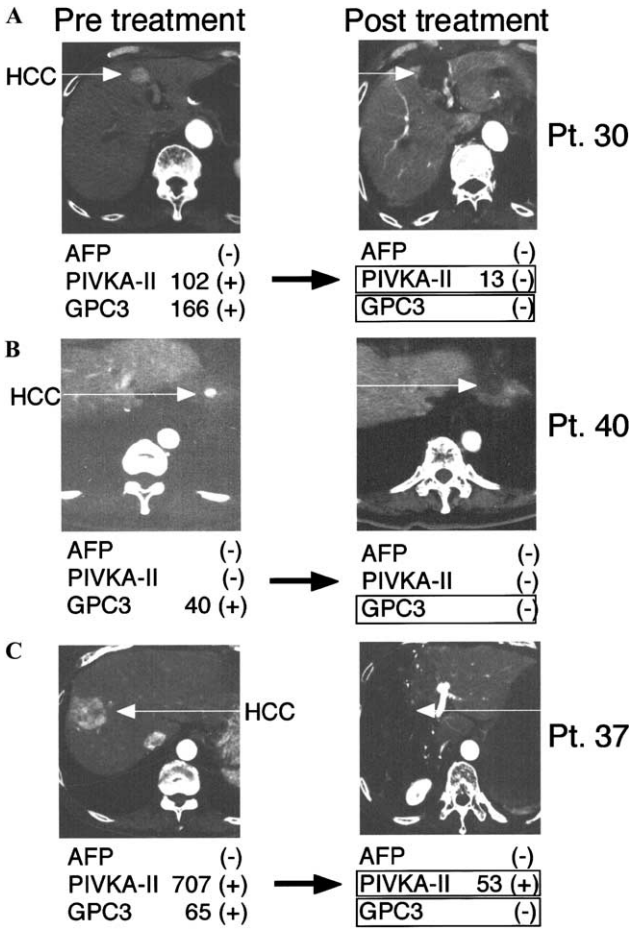

Fig. 4. Disappearance of soluble GPC3 in patients' sera after the surgical treatments of HCC. Computed tomography (CT) of HCC lesion and serum levels of three kinds of tumor markers, AFP, PIVKA-II, and GPC3 before and after surgical treatments are indicated for patients 30 (A), 40 (B), and 37 (C).

HCC excised from 14 patients with HCC. Seven patients (Pts. 2, 6, 7, 8, 11, 29, and 37) were positive for serum GPC3 (secreting type) and other seven were negative (non-secreting type; Pts. 10, 12, 17, 23, 26, 34, and 35). The expression of GPC3 protein in seven tumors derived from secreting type patients was divided into two patterns. Secreting type-1 pattern (Pts. 2, 7, and 11) showed a much stronger expression of GPC3 protein in HCC cells than in non-cancerous liver cells (Fig. 5). Secreting type-2 pattern (Pts. 6, 8, 29, and 37) showed weak expression of GPC3 protein in HCC cells and showed some expression in non-cancerous liver tissue (Fig. 5). Because GPC3 mRNA isolated from tumor of Pt. 37 showed a higher expression than did that of non-cancerous liver tissue, it was thought that the majority of GPC3 protein in this type of HCC cells was almost secreted away. On the other hand, all seven non-secreting type tumors showed moderate expression of GPC3 protein with a speckled pattern in HCC cells (Fig. 5).

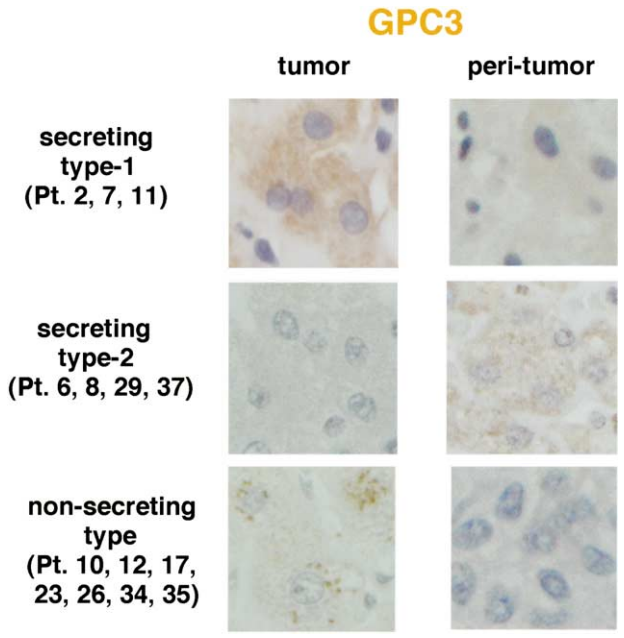

Fig. 5. Immunohistochemical analysis of expression pattern of GPC3 protein in HCC cells. GPC3 immunostaining (colored brown) of sections of tumor (left) and of peri-tumor (right) was indicated at objective magnifications; 400×.

Discussion

In 1996, Pilia et al. reported that *GPC3*, which encodes one member of the glypican family, is mutated in patients with Simpson–Golabi–Behmel syndrome (SGBS) [9]. SGBS is an X-linked disorder characterized by pre- and postnatal overgrowth, and a broad spectrum of clinical manifestations which vary from a very mild phenotype in carrier females to infantile lethal forms in some males [10]. The list of clinical manifestations of SGBS includes a distinct facial appearance, cleft palate, syndactyly, polydactyly, supernumerary nipples, cystic and dysplastic kidneys, congenital heart defects, and so on [11–14]. Most GPC3 mutations are point mutations or small deletions encompassing a varying number of exons [15,16]. Given the lack of correlation between patient phenotype and location of the mutations, it has been proposed that SGBS is caused by the lack of a functional GPC3 protein, with additional genetic factors being responsible for the intra- and interfamilial phenotypic variation [15]. The development of GPC3-deficient mice added a strong support for this hypothesis [17], these mice have several abnormalities found in SGBS patients, including overgrowth, and cystic and dysplastic kidneys.

Because GPC3 is an inhibitor of cell proliferation and can induce apoptosis in certain types of tumor cells [18], reports indicating that GPC3 expression is down-regulated in tumors of different origin were not surprising. Lin et al. [19] showed that, although GPC3 is expressed in the normal ovary, the expression is undetectable in any significant proportion of ovarian cancer cell lines. In all cases where GPC3 expression was lost, the GPC3 promoter was hypermethylated, and mutations were nil in the coding region. GPC3 expression was restored by treatment with a demethylating agent. In addition, the authors demonstrated that ectopic expression of GPC3 inhibits colony-forming activity in several ovarian cancer cell lines. Other data associating GPC3 with cancer were derived from a differential mRNA display study on normal rat mesothelial cells and mesothelioma cell lines [20]. In this study GPC3 was consistently down-regulated in tumor cell lines and a similar down-regulation was noted in primary rat mesotheliomas and in cell lines derived from human mesotheliomas. Similar to cases of ovarian cancer, mutations in the GPC3 coding sequence have not been found, but most cell lines had an aberrant methylation in the GPC3 promoter region. As reported [18], the study showed that ectopic expression of GPC3 in mesothelioma cell lines inhibits colony-forming activity. Xiang et al. [21] reported that GPC3 expression was also silenced in cases of human breast cancer. Collectively, these data suggest that GPC3 can act as a negative regulator of growth in these cancers. Insomuch as the expression of GPC3 is reduced during tumor progression in cancers originating from tissues that are GPC3-positive in adults and this reduction seems to play a role in generation of the malignant phenotype.

On the contrary, in the case of HCC, tumors originating from tissues that express GPC3 only in the embryo, GPC3 expression tends to reappear with malignant transformation. In this study, more than 80% of 27 HCC tumors showed a much stronger expression of GPC3 mRNA than did non-cancerous liver tissue, and immunohistochemical analysis revealed that HCC expressed GPC3 protein in all 14 HCC patients tested. On the other hand, GPC3 protein was positive in sera of 40.0% (16/40) of HCC patients. There was a discrepancy between GPC3 expression and GPC3 secretion. We could classify three GPC3 protein expression patterns in HCC (secreting type1, 2, and non-secreting type). Further investigations are needed to determine why serum GPC3 was detected in only 40% of our HCC patients. Furthermore, whether GPC3 re-expression plays a role in progression of these tumors is unknown. During the last few years it has been clearly established that cell-surface heparan sulfate proteoglycans (HSPGs) are required for the optimal activity of heparin-binding growth factors, such as fibroblast growth factors (FGFs) and Wnts [22,23]. Glypicans are a family of GPI-anchored cell surface HSPGs. We speculate that tissue-specific differences in the relationship between oncogenesis and the expression level of GPC3 are due to the fact that GPC3 may regulate growth and survival factors differently in each tissue.

GPC3 seems to behave in these organs, at least as an oncofetal protein. In general, oncofetal proteins do not seem to play a critical role in tumor progression but have been used as tumor markers or as targets for immunotherapy [24,25]. Whether or not the oncofetal behavior of GPC3 can be tested clinically and whether re-expression of this glypican plays a role in the progression of HCC are under investigation.

AFP and PIVKA-II [26] are well known major tumor markers for HCC. Generally, AFP shows high sensitivity but also high false-positivity. Serum AFP levels are often increased in patients with benign liver diseases, such as CH and LC, when AFP is detected using a more sensitive method. *Lens culinaris* agglutinin-reactive fraction of α-fetoprotein (AFP-L3%) is a recently described marker of HCC. AFP-L3% shows a much higher specificity than AFP, but a lower sensitivity. On the other hand, PIVKA-II shows a lower false-positivity, but is not always sensitive enough to detect small HCCs. In our study, the sensitivity of AFP, AFP-L3%, PIVKA-II, and GPC3 was 20/40 (50%), 10/36 (27.7%), 20/40 (50%), and 16/40 (40%), respectively. We could not diagnose 12 of 40 (30%) HCC patients using AFP and PIVKA-II. Although, we could diagnose an additional four patients as cases of HCC among 12 patients, three were classified as being in a relatively early UICC Stage II, hence GPC3 may be useful for diagnosis of early stage HCC. We could diagnose 80% of our patients with HCC using AFP, PIVKA-II, and the novel tumor marker, GPC3. Furthermore, GPC3 protein in the sera was detectable only in HCC patients and not in patients with other liver diseases or other kinds of cancers and healthy donors, thereby indicating that the specificity is 100%. Furthermore, we confirmed that GPC3 protein had disappeared from the sera of three patients after surgical treatments for HCC. Taken together, these results indicate that GPC3, as defined in our study, may prove to be an appropriate candidate for use in cancer-diagnosis for large numbers of patients with HCC.

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