Identification of Glypican-3 as a Novel Tumor Marker for Melanoma

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ABSTRACT

Purpose: **We reported recently the novel tumor marker glypican-3 (GPC3) for hepatocellular carcinoma. In the present study, we investigated the expression of *GPC3* in human melanoma cell lines and tissues and asked whether GPC3 could be a novel tumor marker for melanoma.**

Experimental Design: **Expression of GPC3 mRNA and protein was investigated in human melanoma cell lines and tissues using reverse transcription-PCR and immunohistochemical analysis. Secreted GPC3 protein was quantified using ELISA in culture supernatants of melanoma cell lines and in sera from 91 patients with melanoma and 28 disease-free patients after surgical removal of primary melanoma. All of the subjects were Japanese nationals.**

Results: **In >80% of melanoma and melanocytic nevus, there was evident expression of *GPC3* mRNA and protein. Furthermore, GPC3 protein was evidenced in sera of 39.6% (36 of 91) of melanoma patients but not in sera from subjects with large congenital melanocytic nevus (0 of 5) and from healthy donors (0 of 60). Twenty-seven of 36 serum GPC3-positive patients were negative for both serum 5-S-cysteinyldopa and melanoma-inhibitory activity, well-known tumor markers for melanoma. The positive rate of serum GPC3 (39.6%) was significantly higher than that of 5-S-cysteinyldopa (26.7%) and of melanoma-inhibitory activity (20.9%). Surprisingly, we detected serum GPC3 even in patients with stage 0 *in situ* melanoma. The positive rate of serum GPC3 at stage 0, I, and II (44.4%, 40.0%, and 47.6%) was significantly higher than that of 5-S-cysteinyldopa (0.0%, 8.0%, and 10.0%). Also observed was the disappearance of GPC3 protein in sera from 11 patients after surgical removal of the melanoma.**

Conclusions: **GPC3 is apparently a novel tumor marker useful for the diagnosis of melanoma, especially in early stages of the disorder.**

INTRODUCTION

The incidence of melanoma is increasing worldwide. In the last decade, several molecules have been evaluated as tumor markers to detect melanoma, including melanin metabolites, adhesion molecules, cytokines, and melanoma-associated antigens [reviewed by Brochez and Naeyaert (1), Hauschild *et al.* (2), and Hartleb and Arndt (3)]. Several investigators reported that 5-S-cysteinyldopa is useful as a marker for melanoma progression or for monitoring metastatic melanoma (4–7). 5-S-cysteinyldopa is usually used as tumor marker for melanoma in Japan, and the usefulness of melanoma-inhibitory activity was reported (8, 9). However, current methods are not sensitive enough to detect organ metastasis at such early stages. There is no available tumor marker that can detect primary melanoma at early stages, with a small size and without metastasis. A simple, inexpensive, and noninvasive method to detect a serum tumor marker would aid the management of high-risk patients.

We recently identified glypican-3 (GPC3) overexpressed specifically in human hepatocellular carcinoma, as based on cDNA microarray data, and we reported that GPC3 is a novel tumor marker for hepatocellular carcinoma (10). Soluble GPC3 protein was detected in sera of hepatocellular carcinoma patients but not in case of other liver diseases or cancers other than melanoma. We also accidentally detected *GPC3*-expression in a B16 mouse melanoma cell line. This observation prompted us to examine the expression of GPC3 in human melanoma, and we asked whether GPC3 might be a novel tumor marker for melanoma. Indeed, GPC3 was detected in the sera of 40% patients with melanoma, irrespective of clinical stages and even in the sera of patients with stage 0 *in situ* melanoma.

MATERIALS AND METHODS

Melanoma and Melanocytic Nevus Tissues, Blood Samples, and Cell Lines. After receiving informed written consent, we obtained tissue and blood samples from melanoma and melanocytic nevus patients treated in the Department of Dermatology, Graduate School of Medical Sciences, Kumamoto University (Kumamoto, Japan). They were stored at −80°C until use. We collected patient profiles from medical records to determine the clinical stages, according to the Unio Internationale Contra Cancrum/American Joint Committee on Cancer Tumor-Node-Metastasis classification (11).

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Ninety-one consecutive and preoperative patients with melanoma consisted of 43 men and 48 women with an average age of 65.7 years (range, 22 to 89 years); 9 had stage 0 (*in situ*); 25 had stage I; 21 had stage II; 18 had stage III; and 18 had stage IV melanoma. Twenty-eight disease-free patients after removal of primary lesions consisted of 15 men and 13 women; One had stage 0; 8 had stage I; 14 had stage II; and 5 had stage III melanoma. All of the patients were Japanese nationals.

Melanoma cell lines CRL1579, G361, HMV-I, and SK-MEL-28 were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan), and 888mel and 526mel were provided by Dr. Yutaka Kawakami, Keio University (Tokyo, Japan). HMV-I, SK-MEL-28, Ihara, and MeWo were cultured in DMEM supplemented with 10% fetal calf serum, and CRL1579, G361, 888mel, 526mel, 164, SK-MEL-19, and Colo38 were cultured in RPMI 1640 supplemented with 10% fetal calf serum. Human epidermal melanocytes, neonatal, in culture medium 154S supplemented with human melanocyte growth supplement were purchased from KURABO (Osaka, Japan).

Reverse Transcription-PCR. Total RNA was isolated from homogenized tissues and cell lines using the TRIZOL Reagent (Life Technologies, Inc., Rockville, MD). Reverse transcription-PCR (RT-PCR) was done, as described (12). We designed *GPC3* gene-specific PCR primers to amplify fragments of 939 bp, and we used RT-PCR reactions consisting of initial denaturation at 94°C for 5 minutes, 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 tissues and cell lines.

Immunohistochemical Examination and ELISA. Immunohistochemical examinations were done, as described (13, 14). We stained sections with antihuman GPC3 303–464 antibodies (H-162; Santa Cruz Biotechnology, Santa Cruz, CA). For the negative control, staining replaced the primary antibody with an immunoglobulin fraction from preimmune rabbit serum. The percentage of stained cells in each section was estimated independently by two observers (T. K. and T. O.). ELISA of GPC3 was done as described (10). In ELISA method-1, we used the same anti-GPC3 antibody (H162) and its biotinylated one. To independently confirm the accuracy of this ELISA system for specific detection of GPC3, we used another antihuman GPC3 goat polyclonal antibody (W-18) raised against a NH_2-terminal peptide (Santa Cruz Biotechnology) and used this antibody for ELISA detection of serum GPC3. Sandwich ELISA method-2 was performed by using W-18 fixed on the solid surface and biotinylated H-162.

Statistical Analysis. We analyzed all of the data using the StatView statistical program for Macintosh (SaS, Cary, NC), then evaluated the statistical significance using Student's *t* test, χ^2, and Fisher's exact test. We considered Ps < 0.05 to be statistically significant.

RESULTS

Expression of *GPC3* mRNA in Human Melanoma. We examined expression of *GPC3* mRNA using RT-PCR. Expression of *GPC3* mRNA was evidenced in 8 of 11 human melanoma cell lines (Fig. 1*A*). 164, 888mel, Ihara, CRL1579, and MeWo melanoma cell lines showed stronger expression of *GPC3* mRNA than did 526mel, G361, and SK-MEL-28, whereas SK-MEL-19, Colo38, and HMV-I showed no such expression. Primary tumor of melanoma from patients 50, 65, 78, 71 (Fig. 1*B*), and 68 (data not shown), and lymph node metastasis of patient 65 (Fig. 1*B*) showed positive expression, whereas normal skin, including a few melanocytes, showed no such expression (Fig. 1*B*). On the contrary, cultured human neonatal epidermal melanocytes showed moderate expression of *GPC3* mRNA. Tissues of melanocytic nevus also showed positive expression (Fig. 1*B*). Hence, all of the tissues of melanoma and melanocytic nevus we checked showed positive *GPC3* mRNA expression.

Expression of GPC3 Protein in Human Melanoma Tissues. An immunohistochemical analysis of GPC3 was made on 21 primary melanomas and 11 melanocytic nevus tissues. The results of immunostaining are classified by the percentage of stained cells: +++, >75%; ++, 50% to 75%; +, 25% to 50%; ±, <25%; −, negative. The results in melanoma are summarized in Table 1, and representative staining is shown in Fig. 2. Many cases of primary melanoma lesions (17 of 21, 81.0%) showed expression of GPC3 protein in melanoma cells (+++, 6; ++, 6; +, 0; ±, 5; −, 4 cases; Table 1, Fig. 2, *A* and *B*). Ten of 11 melanocytic nevus lesions also showed positive expression (+++, 4; ++, 4; +, 1; ±, 1; −, 1 cases; Fig. 2*C*).

The Presence of Soluble GPC3 Protein in Culture Supernatants of Melanoma Cell Lines and Sera from Melanoma Patients. We next detected soluble GPC3 using ELISA method-1. 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 supports the accuracy of ELISA (data not shown). We defined the con-

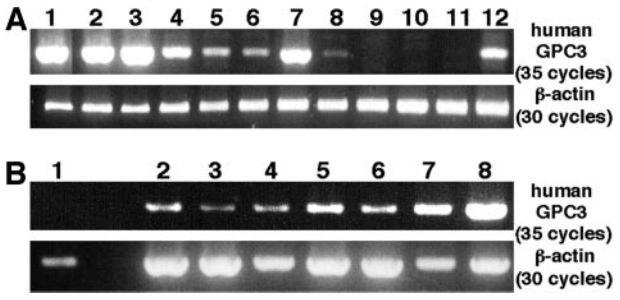

Fig. 1 Expression of *GPC3* mRNA in human melanoma, melanocyte, and melanocytic nevus. *A*, expression of *GPC3* mRNA detected using RT-PCR in human melanoma cell lines (*Lanes 1–11*) and neonatal epidermal melanocytes (HEMn; *Lane 12*). *Lanes 1*: 164, *2*: 888mel, *3*: Ihara, *4*: CRL1579, *5*: 526mel, *6*: G361, *7*: MeWo, *8*: SK-MEL-28, *9*: SK-MEL-19, *10*: Colo38, *11*: HMV-I, *12*: HEMn. *B*, expression of *GPC3* mRNA detected using RT-PCR in human tissues of normal epidermis (*Lane 1*), melanoma (*Lanes 2–6*), and melanocytic nevus (*Lanes 7* and *8*). Primary melanoma tissues originated from patient 50: *Lane 2*, patient 65: *Lane 3*, patient 78: *Lane 5*, patient 71: *Lane 6*, and tissue of metastasis to lymph node of patient 65 is shown in *Lane 4*.

Table 1 Profiles of 91 Japanese patients with melanoma, and quantification of GPC3, 5-S-CD, and MIA in sera of patients

							Serum concentrations of tumor markers			
					GPC3 expression		GPC3 (U/ml)			
Pt. ID	Stage*	Age	Sex	Type	mRNA†	Protein‡	Method 1§	Method 2¶	5-S-CD (nmol/L)‖	MIA (ng/ml)**
1	0	60	M	SSM			0	0	4.6	12.3
2	0	64	F	LM			0	0	2.8	9.8
3	0	78	F	ALM			0	0	2.9	14.1
4	0	74	M	LM			0	0	6.2	19.4
5	0	85	F	ALM		+++	25	24	3.2	9.5
6	0	72	M	LM			0	0	3.8	14.5
7	0	48	F	ALM			38	41	3.3	8.4
8	0	69	F	ALM			22	26	2.6	10.0
9	0	66	F	ALM			8	4	4.7	12.2
10	IA	33	M	MUCOUS		±	103	104	1.9	7.8
11	IA	82	F	LMM			40	41	5.5	17.2
12	IA	75	F	SSM		±	25	19	3.9	16.5
13	IA	41	F	SSM		+++	20	13	2.3	6.8
14	IA	70	F	MUCOUS			0	0	2.4	12.1
15	IA	78	M	ALM			0	0	11.0	17.8
16	IA	60	F	ALM		±	0	0	3.6	16.4
17	IA	61	M	ALM			0	0	1.0	10.1
18	IA	62	F	ALM			0	0	5.9	10.6
19	IA	73	M	ALM			0	0	3.6	10.8
20	IA	70	M	LMM			20	21	8.0	16.2
21	IA	33	F	MUCOUS			10	15	3.6	8.2
22	IA	66	M	ALM			0	0	5.9	13.7
23	IA	76	M	ALM			0	0	5.8	18.5
24	IA	58	F	ALM			0	0	4.4	10.8
25	IA	89	M	LMM			0	0	47.6	17.4
26	IB	58	F	ALM			61	98	2.5	8.9
27	IB	58	F	MUCOUS		−	0	0	8.6	13.4
28	IB	66	F	MUCOUS			23	44	2.9	13.6
29	IB	56	F	SSM			10	11	2.3	13.8
30	IB	64	F	ALM			20	8	7.5	15.5
31	IB	84	F	ALM			0	0	2.0	8.9
32	IB	79	M	ALM		+++	0	0	7.3	18.7
33	IB	76	F	ALM			0	0	5.7	16.3
34	IB	74	F	ALM		++	0	0	2.8	14.7
35	IIA	74	F	SSM			106	108	17.4	10.5
36	IIA	75	M	ALM			54	61	NT††	13.5
37	IIA	74	F	ALM			16	8	3.4	12.5
38	IIA	64	M	ALM		±	0	0	4.6	10.7
39	IIA	47	F	SSM			0	0	4.3	14.0
40	IIA	77	F	LMM			34	39	3.9	9.4
41	IIB	50	F	SSM			75	59	6.4	15.3
42	IIB	72	M	LMM			73	66	7.0	13.1
43	IIB	88	M	ALM		++	0	0	1.2	12.8
44	IIB	63	M	ALM		−	0	0	3.7	11.2
45	IIB	77	M	SSM			0	0	NT	11.8
46	IIB	69	M	ALM			15	16	4.6	9.9
47	IIB	57	M	ALM			0	0	3.4	17.1
48	IIB	69	F	ALM			0	0	3.3	14.7
49	IIB	71	M	ALM		++	0	0	4.7	7.3
50	IIC	79	F	ALM	+		25	30	3.8	6.2
51	IIC	42	M	SSM		−	0	0	6.3	7.9
52	IIC	72	F	ALM			0	0	3.6	12.2
53	IIC	75	F	MUCOUS			0	0	8.7	13.0
54	IIC	77	M	ALM			16	19	7.3	10.9
55	IIC	83	M	SSM			10	9	13.3	11.3
56	IIIA	83	M	ALM		++	0	0	7.7	19.5
57	IIIA	55	M	ALM			0	0	8.2	10.2
58	IIIA	86	F	ALM		++	0	0	9.7	12.5
59	IIIA	79	F	ALM		+++	0	0	6.1	15.9
60	IIIA	70	M	ALM			0	0	4.0	14.8
61	IIIA	63	F	SSM			10	7	11.8	12.1
62	IIIA	79	M	NM			10	10	4.0	14.9
63	IIIA	53	F	MUCOUS			0	0	5.2	13.7
64	IIIB	85	M	ALM			140	126	9.2	24.4
65	IIIB	56	M	LMM	+		0	0	15.5	14.9

Table 1 Continued

							Serum concentrations of tumor markers			
					GPC3 expression		GPC3 (U/ml)			
Pt. ID	Stage*	Age	Sex	Type	mRNA†	Protein‡	Method 1§	Method 2¶	5-S-CD (nmol/L)‖	MIA (ng/ml)**
66	IIIB	59	M	MUCOUS			0	0	1.2	7.0
67	IIIB	77	M	ALM			67	85	7.1	12.1
68	IIIC	35	F	NM	+		132	130	8.4	6.3
69	IIIC	63	F	ALM		±	18	8	4.9	14.0
70	IIIC	50	F	unknown			0	0	5.9	28.6
71	IIIC	47	M	MUCOUS	+		0	0	10.3	5.9
72	IIIC	70	M	ALM		−	22	20	24.2	11.4
73	IIIC	63	M	ALM			0	0	14.4	15.1
74	IV	47	F	SSM		++	35	37	12.7	86.0
75	IV	77	M	ALM			0	0	748	102
76	IV	65	M	unknown			0	0	492	23.1
77	IV	78	M	MUCOUS			0	0	44.6	14.6
78	IV	60	F	SSM	+		0	0	32.4	26.8
79	IV	76	F	MUCOUS			0	0	1.1	9.0
80	IV	72	F	SSM			0	0	981	438
81	IV	73	F	SSM			0	0	56.1	7.7
82	IV	45	F	unknown			10	5	5.5	11.6
83	IV	60	F	MUCOUS			8	13	8.6	11.2
84	IV	72	M	NM			0	0	225	412
85	IV	50	M	SSM			0	0	957	438
86	IV	47	F	NM		+++	0	0	257	419
87	IV	22	M	unknown			57	60	25.8	10.8
88	IV	39	M	NM			0	0	170	15.8
89	IV	74	F	ALM			0	0	395	22.9
90	IV	68	M	unknown			34	73	74.2	11.9
91	IV	66	F	ALM		+++	0	0	246	9.2

Abbreviations: ALM, acral lentiginous melanoma; SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma; UICC, Unio Internationale Contra Cancrum; AJCC, American Joint Committee on Cancer; TNM, Tumor-Node-Metastasis; MIA, melanoma-inhibitory activity.

* Clinical stages, according to the UICC/AJCC TNM classification (11).

† The expression of *GPC3* mRNA detected by RT-PCR as shown in Fig. 1*B*.

‡ The expression of GPC3 protein detected by immunohistochemical analysis as shown in Fig. 2. The results of immunostaining are symbolized by the positive rate of all stained melanoma cells: +++, >75%; ++, 50% to 75%; +, 25% to 50%; ±, <25%; −, negative.

§ Soluble GPC3 protein in the sera was quantified by ELISA method 1 using polyclonal anti-GPC3 antibody (H-162). We could obtain reproducible results by using three different batches of antibodies H-162 and representative results were shown. We arbitrarily fixed the cut-off value at 1 unit/mL, and positive values are underlined.

¶ Soluble GPC3 protein in the sera was quantified by sandwich ELISA method 2 using goat polyclonal anti-GPC3 antibody (W-18) raised against a NH_2-terminal peptide of GPC3 and biotinilated H-162. We arbitrarily fixed the cut-off value at 1 unit/mL and positive values are underlined.

‖ 5-S-CD was quantified using high performance liquid chromatography by us. The cut-off value was fixed at 10 nmol/L (6), and positive values are underlined.

** MIA in the sera was detected by ELISA. We arbitary fixed the cut-off value at 17 ng/mL in this study, and positive values are underlined.

†† Not tested.

centration of GPC3 protein in the 1 mL of the culture supernatant of 1×10^5 HepG2 cells after cultivation for 24 hours as 1 units/mL. Soluble GPC3 protein could be detected in culture supernatants of 5 of 11 melanoma cell lines (Fig. 3*A*). The amount of GPC3 protein in the culture supernatants of the 164 was larger than that of the SK-MEL-28, 526mel, G361, and CRL1579. On the other hand, GPC3 was not detected in the 888mel, Ihara, and MeWo (Fig. 3*A*), despite the strong expression of *GPC3* mRNA (Fig. 1*A*). Thus, there was some discrepancy between *GPC3* mRNA expression in melanoma cell lines and the amount of GPC3 protein secreted from these cells into culture supernatants. For example, we did not detect soluble GPC3 protein in the culture supernatant of human epidermal melanocytes, despite the expression of mRNA (Fig. 1*A*).

The quantification by ELISA method-1 of GPC3 protein in sera of 91 preoperative patients with melanoma, 5 patients with large congenital melanocytic nevus, and of 60 healthy donors who have many small melanocytic nevus is indicated in Fig. 3*B* and Table 1. We detected and quantified GPC3 protein in the sera of 36 of 91 melanoma patients (39.6%) but, more importantly, not in sera of patients with large congenital melanocytic nevus and healthy donors, whereas *GPC3* mRNA and protein were expressed in melanocytic nevus tissues. We could obtain reproducible results by using three different batches of polyclonal anti-GPC3 antibody (H-162) indicating that ELISA detection of soluble GPC3 was not dependent on a particular batch of H-162. We arbitrary fixed the cutoff level at 1 units/mL, because all of the healthy donors were completely negative for serum GPC3, and there was no gray area between GPC3-positive and negative patients.

Furthermore, to confirm these results, we performed sandwich ELISA method 2 by using another antihuman GPC3 goat

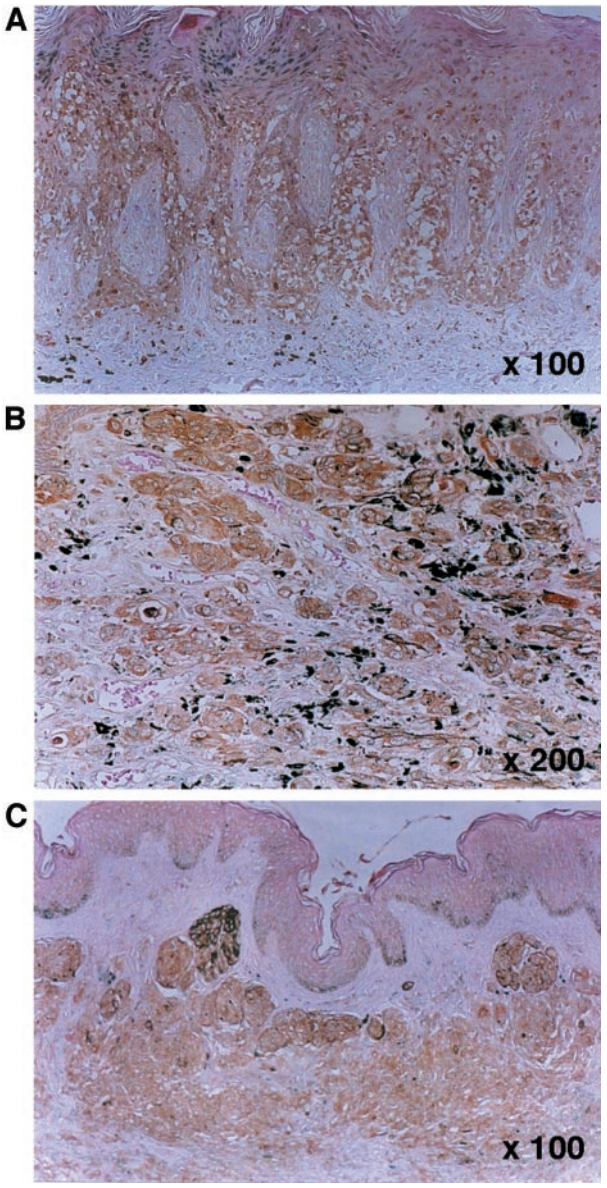

Fig. 2 Expression pattern of GPC3 protein in primary melanoma and melanocytic nevus lesions examined by immunohistochemical staining. *A*, primary melanoma of patient 13; GPC3 immunostaining colored brown was evident in the melanoma cells. *B*, primary melanoma of patient 69; Observation under higher magnification revealed that GPC3 immunoreactivity in melanoma cells was localized mainly in the cytoplasm. *C*, melanocytic nevus (Fig. 1*B*, *Lane 8*) with expression of GPC3. Objective magnifications; *A* and *C*: ×100, *B*: ×200.

polyclonal antibody (W-18) and biotinylated anti-GPC3 303–464 polyclonal antibody (H-162; Fig. 3*C*; Table 1). The results obtained by using these two antibodies were similar ($R^2 = 0.89$) to those obtained by using H-162 alone (method 1), indicating that the detection of serum-soluble GPC3 was not solely dependent on the particular polyclonal antibody H-162. Thus, there was no discrepancy in identification of serum GPC3-positive patients between methods 1 and 2.

The prevalence of GPC3 protein in the sera of melanoma patients was significantly higher than that in other donors ($P < 0.0001$). Although Fig. 1*B* shows that melanoma tumor from patients 50, 65, 78, and 71 expressed *GPC3* mRNA, GPC3 protein was detected only in the serum of patient 50 among these 4 patients. There was no correlation between concentrations of serum GPC3 and its mRNA expressions in the melanoma tissues. There was also no correlation between concentrations of serum secreted GPC3 and levels of GPC3 protein expressed in the tissues.

Among the 21 cases in which immunohistochemical staining of melanoma tissue was done, serum GPC3 was detected in 7 (33.3%) but not in 14 (66.7%; Table 1). In 6 of the 7 (patients 5, 10, 12, 13, 69, and 74), GPC3 protein expression was detected both in the sera and in their melanoma cells, but in the remaining 1 case (patient 72), GPC3 protein expression was detected only in the sera not in melanoma cells. It was thought that almost all of the GPC3 protein produced in melanoma cells of this patient 72 was secreted. On the contrary, in 11 of the 14 (78.6%), serum GPC3 was not detected, despite GPC3 protein expression in their melanoma cells. Only 3 of the 21 cases (14.3%) did not show expression of GPC3 protein in both in melanoma cells and the sera. These results showed that most of melanoma tissues (85.7%) expressed GPC3 protein, and in ~50% of those patients, GPC3 protein was secreted and detected in their sera. On the contrary, although GPC3 was evidenced in most of melanocytic nevus and neonatal epidermal melanocytes, GPC3 protein was not secreted from all of the melanocytic nevus tested and adult epidermal melanocytes.

Comparison of Serum Concentrations of GPC3, 5-S-Cysteinyldopa, and Melanoma-Inhibitory Activity in Patients with Melanoma Classified by Stage. The above results clearly indicated that GPC3 might be a novel tumor marker for melanoma. We next compared the serum concentrations of GPC3, 5-S-cysteinyldopa, and melanoma-inhibitory activity in patients with melanoma classified by stage (Fig. 4; Tables 1 and 2). Fig. 4 shows serum concentrations of GPC3 quantified by ELISA method-1 (Fig. 4*A*), 5-S-cysteinyldopa (Fig. 4*B*), and melanoma-inhibitory activity (Fig. 4*C*) in 91 patients with melanoma (+) and 28 disease-free patients without detectable melanoma (−) classified by stage. We arbitrary fixed the cutoff level at 1 units/ml in GPC3, at 10 nmol/L in 5-S-cysteinyldopa (6), and at 17 ng/mL in melanoma-inhibitory activity, and there were two 5-S-cysteinyldopa false-positive cases in disease-free (−) stage II. Although serum concentrations of 5-S-cysteinyldopa and melanoma-inhibitory activity increased markedly in patients at stage IV, percentages of serum GPC3-positive patients were almost equal among the five clinical stages. To our surprise, we detected GPC3 in the sera of patients with very small melanoma such as stage 0 or I. There was no correlation between the positive state of three tumor markers, GPC3, 5-S-cysteinyldopa, and melanoma-inhibitory activity (Table 1). More importantly, 27 of 36 GPC3-positive patients were negative for both 5-S-cysteinyldopa and melanoma-inhibitory activity (patients 5, 7, 8, 9, and so on), and many were classified as cases of relatively early Unio Internationale Contra Cancrum stages 0, I, and II (Table 1). The positive rate of these three tumor markers in patients with melanoma, classified by stage, is shown in Table 2. Total positive rate of GPC3 (36 of 91, 39.6%) was significantly higher than that of 5-S-cysteinyldopa (26.7%)

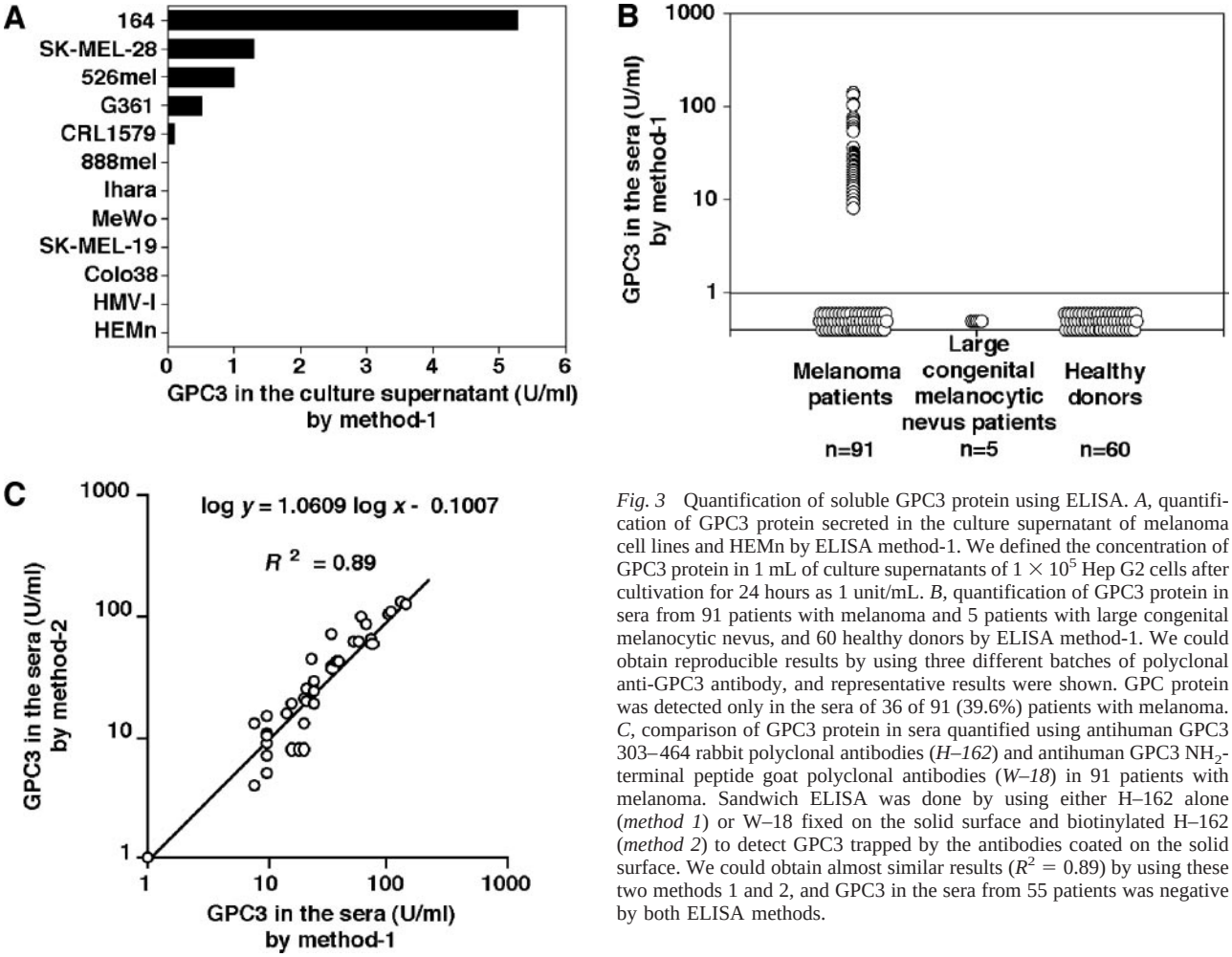

Fig. 3 Quantification of soluble GPC3 protein using ELISA. *A*, quantification of GPC3 protein secreted in the culture supernatant of melanoma cell lines and HEMn by ELISA method-1. We defined the concentration of GPC3 protein in 1 mL of culture supernatants of 1×10^5 Hep G2 cells after cultivation for 24 hours as 1 unit/mL. *B*, quantification of GPC3 protein in sera from 91 patients with melanoma and 5 patients with large congenital melanocytic nevus, and 60 healthy donors by ELISA method-1. We could obtain reproducible results by using three different batches of polyclonal anti-GPC3 antibody, and representative results were shown. GPC protein was detected only in the sera of 36 of 91 (39.6%) patients with melanoma. *C*, comparison of GPC3 protein in sera quantified using antihuman GPC3 303–464 rabbit polyclonal antibodies (*H–162*) and antihuman GPC3 NH_2-terminal peptide goat polyclonal antibodies (*W–18*) in 91 patients with melanoma. Sandwich ELISA was done by using either H–162 alone (*method 1*) or W–18 fixed on the solid surface and biotinylated H–162 (*method 2*) to detect GPC3 trapped by the antibodies coated on the solid surface. We could obtain almost similar results ($R^2 = 0.89$) by using these two methods 1 and 2, and GPC3 in the sera from 55 patients was negative by both ELISA methods.

and melanoma-inhibitory activity (20.9%; $P < 0.01$). Positive rate of GPC3 at stage 0 (4 of 9, 44.4%) was significantly higher than that of 5-S-cysteinyldopa (0.0%; $P < 0.05$), that at stage I (10 of 25, 40.0%) was significantly higher than that of 5-S-cysteinyldopa (8.0%; $P < 0.01$), and that at stage II (10 of 21, 47.6%) was significantly higher than that of 5-S-cysteinyldopa (10.0%) and melanoma-inhibitory activity (4.8%; $P < 0.01$). On the contrary, the positive rate of 5-S-cysteinyldopa at stage IV (15 of 18, 83.3%) was significantly higher than that of GPC3 (27.8%) and melanoma-inhibitory activity (50.0%; $P < 0.01$), and positive rate of melanoma-inhibitory activity at stage IV was significantly higher than that of GPC3 ($P < 0.05$).

Comparison of the Positive Rate of Serum GPC3 in Patients with Melanoma Classified by Clinical Type. We used sera from Japanese patients only in this study. Japanese melanoma has a high frequency of acral lentiginous melanoma, whereas superficial spreading melanoma and lentigo maligna melanoma are frequent types in Caucasians. Some groups have reported that acral lentiginous melanoma differs from other types of melanomas in clinical and histopathological characteristics (15–18). In fact, among 91 melanoma patients investigated in this study, 44 had acral lentiginous melanoma, 16 had superficial spreading melanoma, 9 had lentigo maligna melanoma, 5 had nodular melanoma, 12 had mucous melanoma, and 5 had unknown primary tumors. We next compared the positive rate of serum GPC3 among patients classified by these clinical types (Table 3). There was no significant correlation between the positive rate of serum GPC3 and melanoma type. Therefore, it seems likely that the usefulness of GPC3 as a marker for melanoma is not restricted to Japanese patients.

GPC3 Protein in the Sera of Melanoma Patients Disappeared after Surgical Treatments. Changes in serum levels of three tumor markers, GPC3 quantified by ELISA method-1, 5-S-cysteinyldopa, and melanoma-inhibitory activity, before and after surgical treatments in preoperative GPC3-positive 12 patients (patients 10, 11, 12, 13, 26, 35, 41, 42, 46, 55, 68, and 69) are shown in Table 4. For example, in the case of patient 35, although GPC3 and 5-S-cysteinyldopa were positive in the sera before operation, they disappeared after surgical treatments. GPC3 protein was detected in sera of these 12 patients before surgery but not so after the surgical treatments of patients with melanoma, except for patient 11 who could not be followed after the postoperative day 27. In case of patient 55, although 5-S-cysteinyldopa was weakly positive in serum at postoperative

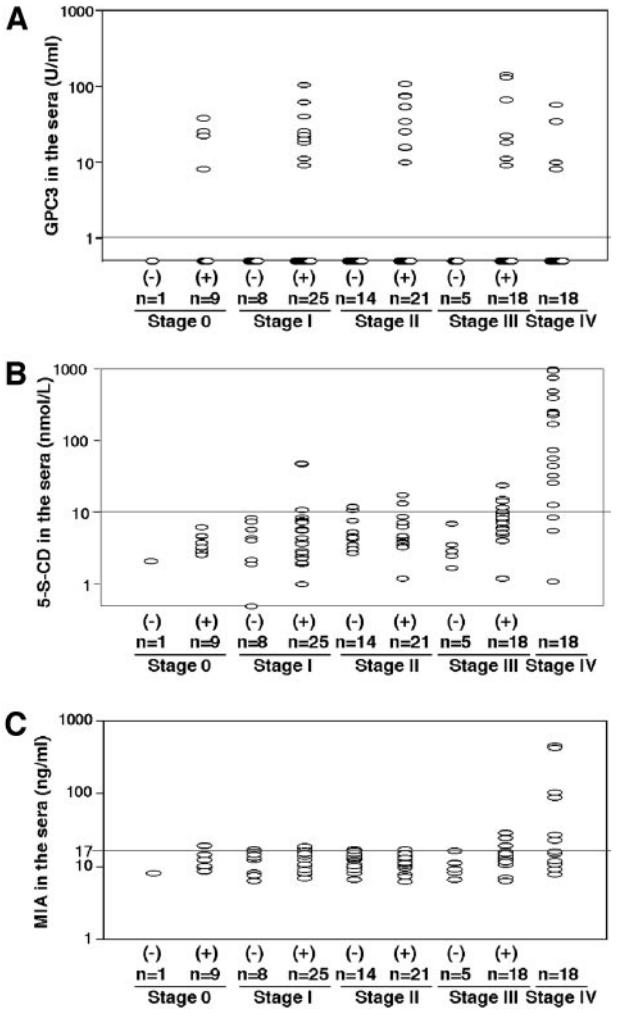

Fig. 4 Comparison of serum concentrations of GPC3, 5-S-CD, and MIA in patients with melanoma classified by stage. *A*, serum concentrations of GPC3 measured by ELISA method-1, (*B*) 5-S-CD, and (*C*) MIA in 91 patients with melanoma (+) and 28 disease-free patients without detectable melanoma (−) classified by stage. We fixed the cutoff level, indicated by a *line* in each panel, at 1 unit/mL in GPC3, at 10 nmol/L in 5-S-CD, and at 17 ng/mL in MIA. MIA, melanoma-inhibitory activity.

day 925, it was not evidenced at the recurrence of the melanoma (clinical data). It must be noted that GPC3 was the only useful tumor marker to follow the efficacy of surgical treatments for patients 12, 13, 26, 41, 42, 46, 68, and 69.

DISCUSSION

There are many tumor markers including carcinoembryonic antigen (19, 20), carbohydrate antigens CA 19–9 (21), and α-fetoprotein (22). Tumor markers have been used in several settings in cancer patients, including screening measures, differentiating malignant from benign lesions, monitoring the response to treatment, and detecting recurrences. In melanoma, several tumor markers have been evaluated for use as prognostic variables, to monitor response to therapy, and to detect recurrence (4–9, 23–25). These markers include 5-S-cysteinyldopa (4–7), melanoma-inhibitory activity (8, 9), ICAM-I (24), and S100 (25, 26) and are useful to detect stage IV metastatic melanoma. However, recurrent disease often cannot be detected at sufficiently early stages, and there is no available tumor marker that can detect primary melanoma at early stages, of a small size, and without metastases.

We found, in this study, that GPC3 could be a novel useful tumor marker for melanoma, especially at early stage, even stage 0 (*in situ*) melanoma. Although we detected no more than 30% of patients with melanoma, using conventional markers, we could diagnose 40% of those, irrespective of clinical stages, by using serum GPC3 as a tumor marker. In our study, the sensitivity of GPC3, 5-S-cysteinyldopa, and melanoma-inhibitory activity was 36 of 91 (39.6%), 24 of 90 (26.7%), and 19 of 91 (20.9%), respectively. We could diagnose only 32 of 91 (35.2%) melanoma patients using 5-S-cysteinyldopa and melanoma-inhibitory activity. But 27 GPC3-positive patients were negative for both 5-S-cysteinyldopa and melanoma-inhibitory activity. Twenty of these 27 patients (74.1%) were classified as being at a relatively early Unio Internationale Contra Cancrum stages 0, I, and II. Therefore, GPC3 is very useful for diagnosis of melanoma at early stages. Finally, we could diagnose 59 of 91 (64.8%) cases of melanoma using 5-S-cysteinyldopa, melanoma-inhibitory activity, and GPC3, a novel tumor marker. Furthermore, GPC3 is superior in specificity to other markers for melanoma. 5-S-cysteinyldopa often gives a false-positivity result. Serum 5-S-cysteinyldopa levels are often increased in patients with a large congenital melanocytic nevus (26). In this study, there were also 2 false-positive cases in disease-free patients. We reported that GPC3 protein in the sera was detect-

Table 2 Positive rates of serum levels of GPC3, 5-S-CD, and MIA in patients with melanoma classified by stage

Stage	GPC3	5-S-CD	MIA
0	4/9 (44.4%)*	0/9 (0.0%)	1/9 (11.1%)
I	10/25 (40.0%)	2/25 (8.0%)	5/25 (20.0%)
II	10/21 (47.6%)	2/20 (10.0%)	1/21 (4.8%)
III	7/18 (38.9%)	5/18 (27.8%)	3/18 (16.7%)
IV	5/18 (27.8%)	15/18 (83.3%)	9/18 (50.0%)
Total	36/91 (39.6%)	24/90 (26.7%)	19/91 (20.9%)

Abbreviations: MIA, melanoma-inhibitory activity

∗ Values significantly higher than others in the same clinical stage group are underlined.

Table 3 Positive rates of serum levels of GPC3 in patients with melanoma classified by clinical type

Type	Positive rate of GPC3 in sera
ALM	15/44 (34.1%)
SSM	9/16 (56.3%)
LMM/LM	4/9 (44.4%)
NM	2/5 (40.0%)
Mucous	3/12 (25.0%)
Total	33/86 (38.4%)

Abbreviations: ALM, acral lentiginous melanoma; SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma.

Table 4 Changes in serum levels of GPC3, 5-S-CD, and MIA, before and after surgical treatments for melanoma in preoperative GPC3-positive 12 patients

Pt. ID*		Pre-op.	POD12	POD63	POD283
	GPC3 (U/ml)†	103‡	17	44	0
10	5-S-CD (nmol/L)	1.9	18.2	3.1	3.0
	MIA (ng/ml)	7.8	6.2	7.1	7.0
			POD27		
	GPC3 (U/ml)	40	83		
11	5-S-CD (nmol/L)	5.5	NT		
	MIA (ng/ml)	17.2	18.4		
			POD13		
	GPC3 (U/ml)	25	0		
12	5-S-CD (nmol/L)	3.9	NT		
	MIA (ng/ml)	16.5	16.5		
			POD150	POD230	
	GPC3 (U/ml)	20	0	0	
13	5-S-CD (nmol/L)	2.3	2.4	1.8	
	MIA (ng/ml)	6.8	7.5	6.1	
			POD100		
	GPC3 (U/ml)	61	0		
26	5-S-CD (nmol/L)	2.5	6.4		
	MIA (ng/ml)	8.9	12.0		
			POD28		
	GPC3 (U/ml)	106	0		
35	5-S-CD (nmol/L)	17.4	5.0		
	MIA (ng/ml)	10.5	11.1		
			POD124	POD240	
	GPC3 (U/ml)	75	0	0	
41	5-S-CD (nmol/L)	6.4	4.0	4.6	
	MIA (ng/ml)	15.3	16.0	13.6	
			POD4	POD160	
	GPC3 (U/ml)	73	0	0	
42	5-S-CD (nmol/L)	7.0	5.3	7.0	
	MIA (ng/ml)	13.1	9.0	9.2	
			POD32	POD1712	
	GPC3 (U/ml)	15	0	0	
46	5-S-CD (nmol/L)	4.6	6.6	4.5	
	MIA (ng/ml)	9.9	13.0	10.8	
			POD925		
	GPC3 (U/ml)	10	0		
55	5-S-CD (nmol/L)	13.3	11.0		
	MIA (ng/ml)	11.3	14.0		
			POD27	POD50	
	GPC3 (U/ml)	132	0	0	
68	5-S-CD (nmol/L)	8.4	NT	NT	
	MIA (ng/ml)	6.3	14.4	7.6	
			POD98		
	GPC3 (U/ml)	18	0		
69	5-S-CD (nmol/L)	4.9	5.7		
	MIA (ng/ml)	14.0	11.6		

Abbreviations: POD, postoperative days; NT, not tested.
* Pt. ID was the same as shown in Table 1.
† Values quantified by ELISA method 1.
‡ Positive values are underlined.

able only in hepatocellular carcinoma patients and not in patients with other liver disease or other kinds of cancers (colon, gastric, pancreatic, biliary, esophageal, lung, and breast; ref. 10), and in this study, GPC3 protein in the sera was detectable in patients with melanoma, but not in disease-free patients after removal of the primary lesion or patients with large congenital melanocytic nevus and healthy donors, thus indicating the specificity of serum GPC3 to be 100% except for patients with hepatocellular carcinoma who were also positive for serum GPC3 as described by us (10) and others (27, 28). We confirmed the disappearance of GPC3 protein in the sera of 11 patients after surgical treatments for melanoma. Thus GPC3 is useful for monitoring the response to treatment. Taken together, these results indicate that GPC3 may prove to be an appropriate candidate for use in making a diagnosis for numbers of patients with melanoma.

There is no convincing correlation among concentrations of secreted GPC3 as measured by ELISA and levels of *GPC3* mRNA expression determined by RT-PCR in melanoma cell lines and melanoma tissues (Fig. 1 and Fig. 3*A*; Table 1). Furthermore, there was no correlation among serum GPC3 levels and GPC3 detected by immunohistochemical analysis in melanoma patients (Table 1). We could classify melanomas into four types in terms of GPC3 protein expression and secretion as follows: secreting type 1, 2, nonsecreting type, and nonexpression type. Secreting type 1 melanoma showed a much stronger expression of GPC3 protein in melanoma cells irrespective of serum GPC3 level (patients 5, 13, and 74). On the contrary, secreting type-2 melanoma showed weak or no expression of GPC3 protein in melanoma cells (patients 10, 12, 69, and 72). Many of nonsecreting type melanoma showed moderate to strong expression of GPC3 protein in melanoma cells. Either way, ~40% of melanoma showed characteristics of secreting GPC3, irrespective of GPC3 expression levels. The same phenomena were also observed in hepatocellular carcinoma as reported by us (10). The mechanisms of secretion of GPC3 from melanoma and hepatocellular carcinoma remain to be elucidated.

There was no significant correlation among GPC3 expression or secretion and disease progression (Table 1; Fig. 4*A*). Many of melanocytic nevus showed moderate to strong expression of GPC3 without secretion. On the contrary, melanoma showed various expression levels of GPC3, and 40% of melanoma secreted GPC3. Thereby GPC3 expression may be important for promotion of melanoma but not for melanoma progression. However, we could not prove in this study whether or not GPC3 expression or secretion was important for melanoma progression. We speculate that GPC3 secretion may depend on the character of the cancer cells, for example, the difference in activity of protease. These questions remain to be investigated. To additionally elucidate the role of GPC3 in melanoma progression, a panel of well-characterized melanoma cell lines, which represent each stage from radial growth phase, vertical growth phase (29), to metastatic melanomas, would be useful.

In 1996, Pilia *et al.* (30) reported that *GPC3*, which encodes one member of the glypican family, is mutated in patients with Simpson-Golabi-Behmel syndrome. This syndrome is an X-linked disorder characterized by pre- and postnatal overgrowth and a broad spectrum of clinical manifestations that vary

from a very mild phenotype in carrier females to infantile lethal forms in some males (31). The list of clinical manifestations of this syndrome includes a distinct facial appearance, cleft palate, syndactyly, polydactyly, supernumerary nipples, cystic and dysplastic kidneys, and congenital heart defects (32, 33). Most *GPC3* mutations are point mutations or small deletions encompassing a varying number of exons (34, 35). Given the lack of correlation between patient phenotype and location of the mutations, it has been proposed that Simpson-Golabi-Behmel syndrome is caused by the lack of a functional GPC3 protein, with additional genetic factors being responsible for the intra- and interfamilial phenotypic variation (28). The development of *GPC3*-deficient mice added a strong support for this hypothesis (36). These mice have several abnormalities found in Simpson-Golabi-Behmel syndrome patients, including overgrowth and cystic and dysplastic kidneys. Furthermore, it was reported that GPC3 could induce apoptosis in certain types of tumor cells (37). Some reports indicated that *GPC3* expression is down-regulated in tumors of different origins. They showed that, although *GPC3* is expressed in normal ovary, mammary gland, and mesothelial cells, the expressions are undetectable in a significant proportion of ovarian, breast cancer, and mesothelioma cell lines (38). In all of the 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 these cancer cell lines. 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, this reduction seems to play a role in generation of the malignant phenotype.

On the contrary, in the case of hepatocellular carcinoma, tumors originating from tissues that express GPC3 only in the embryo, GPC3 expression tends to reappear with malignant transformation. Whether or not re-expression of GPC3 plays a role in progression of these tumors is unknown. Why only in hepatocellular carcinoma and melanoma is GPC3 up-regulated and whether GPC3 is involved in oncogenesis of melanoma and hepatocellular carcinoma are some of our ongoing projects.

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