D

Intra-splenic tumor injection

CD8   x 100   TUNEL   x 200   TUNEL   x 400

Untransferred

BM-DC
+ GPC3-8

E   Brain   Liver   Lung   Heart

HE
x 200

CD8
x 200

F
                                    mammary
              placenta   gland   fetal liver

CD8
x 200

Fig. 4. Continued.

BM-DC+GPC3-8-vaccinated female mice not used for breeding, s.c. inoculation of C26/GPC3 or C26 cells (3×10^4) into the right flank of these female mice was done. All these vaccinated mice completely rejected 3×10^4 of C26/GPC3 cells 100 days after inoculation but not C26 cells (data not shown). Pregnancy and births in vaccinated female mice were normal. Six mice were born from each untreated and vaccinated three female mice, and all neonates were normal. Placenta and mammary gland of BM-DC+GPC3-8-vaccinated female mice and fetal livers had normal structures and cellularity with no pathological changes caused by immune responses, such as lymphocyte infiltration or tissue destruction and repair. There were no CD8^{+} T cells in these tissues, which had been immunohistochemically stained (Fig. 4F). Furthermore, to ascertain induction of GPC3-8-specific CTLs in vaccinated female mice that gave birth, s.c. inoculation of C26/GPC3 or C26 cells (1×10^6) into the right flank of these mice was done. After tumor formation, we removed the tumor and immunohistochemically stained it with anti-CD8 antibody. Infiltration of CD8^{+} T cells into C26/GPC3 tumors but not into C26 tumors, as observed in Fig. 4C, was evidenced (data not shown). These results indicate that in this vaccination model GPC3-8-specific CD8^{+} CTLs do not attack placenta and fetal livers expressing GPC3.

DISCUSSION

In 1996, Pilia *et al.* (35) reported that *GPC3* is mutated in patients with Simpson-Golabi-Behmel syndrome. This syndrome is an X-linked disorder characterized by pre- and post-natal overgrowth (36). *GPC3*-deficient mice have several abnormalities found in Simpson-Golabi-Behmel syndrome patients, including overgrowth and cystic and dysplastic kidneys (37). Some reports indicated that GPC3 expression is down-regulated in tumors of different origin. They showed that, although GPC3 is expressed in the 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 cases where GPC3 expression was lost, the *GPC3* promoter was hypermethylated, and mutations were nil in the coding region. Collectively, these data suggest that GPC3 can act as a negative regulator of growth in these cancers.

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 reexpression of GPC3 plays a role in progression of these tumors is unknown. Why is GPC3 up-

regulated only in hepatocellular carcinoma and melanoma? We are investigating to determine whether GPC3 is involved in the oncogenesis of melanoma and hepatocellular carcinoma.

The genetic approach of T-cell epitope cloning method (2–6) and SEREX method (7–11) led to identification of a many genes encoding for tumor antigens and antigenic peptides recognized by tumor-reactive CTLs, thereby enhancing the possibility of antigen-specific cancer immunotherapy (2–8). MAGE (2) and NY-ESO-1 (8) represent cancer-testis antigen, and MART-1 (39), gp100 (40, 41), and tyrosinase (42) represent melanocyte-differentiation antigen. Cancer-testis antigens, expressed only in tumor cells and not in normal adult tissues, except for immune privilege tissues, including testis, ovary, and placenta, are ideal targets for tumor immunotherapy. One can prevent development of autoimmune diseases by vaccination with cancer-testis antigens, and many cancer-testis antigens are expressed in a variety of cancers (43). However, the rates of expression of cancer-testis antigens in cancers are at most 50% (43), and expression often shows heterogeneity within the same tumor (43, 44).

On the contrary, melanocyte-differentiation antigens are expressed homogeneously in almost all melanoma cells, so one can use these antigens for immunotherapy of melanoma patients. However, autoimmunity, such as vitiligo and uveitis, developed after vaccination with these antigens because these antigens are expressed in normal melanocytes (33). cDNA microarray technology is rapidly progressing (12–16). We have recently succeeded in identifying a novel cancer rejection antigen specifically expressed in esophageal cancer with cDNA microarray technology (17). In the present study, we found that GPC3 is highly immunogenic to stimulate eradication by T cells of tumor expressing GPC3 in mice. GPC3 is an ideal candidate antigen useful for immunotherapy of hepatocellular carcinoma, and vaccination against GPC3 is not expected to induce autoimmune diseases because it has unique tissue specificity regarding protein expression. Our study is the first to show that cDNA microarray technology is useful for identifying ideal cancer antigens.

The *HLA-A24* is the most common *HLA class I* allele in the Japanese population, and 60% of Japanese (95% of whom are genotypically *A*2402*), 20% of Caucasians, and 12% of Africans are positive for *HLA-A24* (45, 46). It is important for especially Japanese to identify HLA-A24–restricted CTL epitope peptides. Structural motifs of peptides bound to human HLA-A24 and BALB/c mouse K^d are similar (47–49), and the amino acid sequences of human and mouse GPC3 have a 95% homology. We searched for GPC3-derived peptides with a common sequence in both and selected peptides carrying binding motifs to both HLA-A24 and K^d molecules, as previously described, and we prepared 12 different synthetic peptides GPC3-1~12. GPC3-derived and K^d- restricted CTL epitope identified in BALB/c mice will be applicable to human HLA-A24–restricted CTLs. Therefore, we selected BALB/c mice in this study. Although we wanted to use hepatocellular carcinoma or melanoma cell lines, these cell lines derived from BALB/c mouse were not available. Furthermore, we found no GPC3-expressing murine tumor cell lines originating from BALB/c mice, and we had already established a system for analysis of mouse tumor immunity *in vivo* with Colon26. Anyway, in this

study, with Colon26/GPC3, we could prove that GPC3 could be a cancer rejection antigen in mice. We recently found out that mouse melanoma cell line B16 expressed GPC3, so we are planning to investigate GPC3-mediated antitumor immunity with B16 in C57BL/6 mouse.

We observed the effects of adoptive transfer of highly selected tumor-reactive T cells directed against GPC3-8 peptide, even against established tumors, without causing autoimmune-related destruction. These results show the possibility of therapy of adoptive transfer of GPC3-specific CTLs for both hepatocellular carcinoma and melanoma. We also showed that GPC3-8 peptide-pulsed BM-DCs can prime GPC3-8–specific T cells *in vivo*, and growth of C26 expressing GPC3 was prevented without inducing autoimmune destruction in both s.c. and intrasplenic tumor injection models. The transfer of GPC3-8 peptide-pulsed BM-DCs showed no efficacy against the established C26G tumor (data not shown). On the contrary, vaccination of mice with GPC3-8 peptide emulsified in complete Freund's adjuvant could prime GPC3-8–specific T cells *in vivo*, but could not reject the challenge of C26/GPC3 (data not shown). These results show that GPC3-8 peptide-pulsed BM-DC therapy prevented the appearance or recurrence of tumors, yet this procedure did not induce regression of an established tumor. We suggest that adoptive transfer of GPC3-specific CTLs is a novel treatment strategy for patients with hepatocellular carcinoma or melanoma, and we also suggest that GPC3-derived peptide-pulsed DC vaccination is a novel strategy for prevention of hepatocellular carcinoma or melanoma in patients treated surgically, in patients with liver cirrhosis and chronic hepatitis who are at high risk for development of hepatocellular carcinoma, and in humans at high risk for development of melanoma. Whether GPC3 is an ideal target for immunotherapy in human hepatocellular carcinoma and melanoma, naturally expressing GPC3, will continue to be investigated in our laboratory.

ACKNOWLEDGMENTS

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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 immunohisto-chemical 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-cysteinyl-dopa 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-cysteinyl-dopa (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).

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 NH2-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. 1A). 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. 1B), and 68 (data not shown), and lymph node metastasis of patient 65 (Fig. 1B) showed positive expression, whereas normal skin, including a few melanocytes, showed no such expression (Fig. 1B). On the contrary, cultured human neonatal epidermal melanocytes showed moderate expression of *GPC3* mRNA. Tissues of melanocytic nevus also showed positive expression (Fig. 1B). 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. 2C).

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-
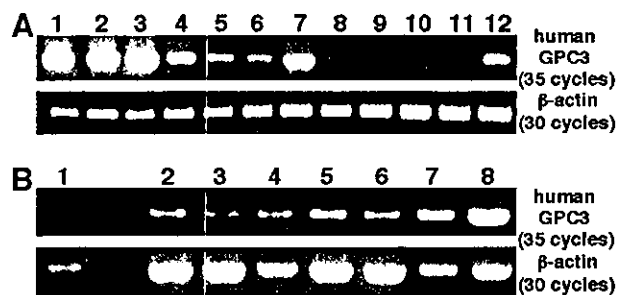
Fig. 1 Expression of *GPC3* mRNA in human melanoma, melanocyte, and melanocytic nevus. *A*, expression of *GPC3* mRNA detected using RT-PCR in human melanoma cell lines (*Lanes 1–11*) and neonatal epidermal melanocytes (HEMn; *Lane 12*). *Lanes 1*: 164, *2*: 888mel, *3*: Ihara, *4*: CRL1579, *5*: 526mel, *6*: G361, *7*: MeWo, *8*: SK-MEL-28, *9*: SK-MEL-19, *10*: Colo38, *11*: HEMn, *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

Pt. ID	Stage*	Age	Sex	Type	GPC3 expression mRNA†	Protein‡	GPC3 (U/ml) 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

Pt. ID	Stage*	Age	Sex	Type	GPC3 expression		GPC3 (U/ml)		5-S-CD (nmol/L)‖	MIA (ng/ml)**
					mRNA†	Protein‡	Method 1§	Method 2¶		
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 NH₂-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 × 10⁵ 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
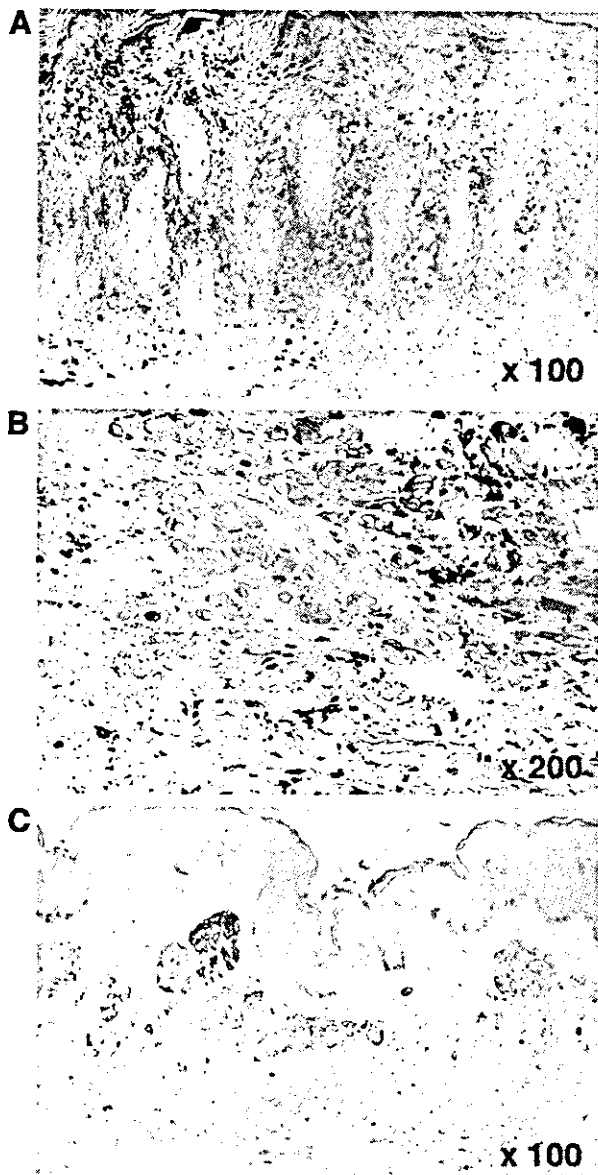
Fig. 2 Expression pattern of GPC3 protein in primary melanoma and melanocytic nevus lesions examined by immunohistochemical staining. *A,* primary melanoma of patient 13; GPC3 immunostaining colored brown was evident in the melanoma cells. *B,* primary melanoma of patient 69; Observation under higher 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%)
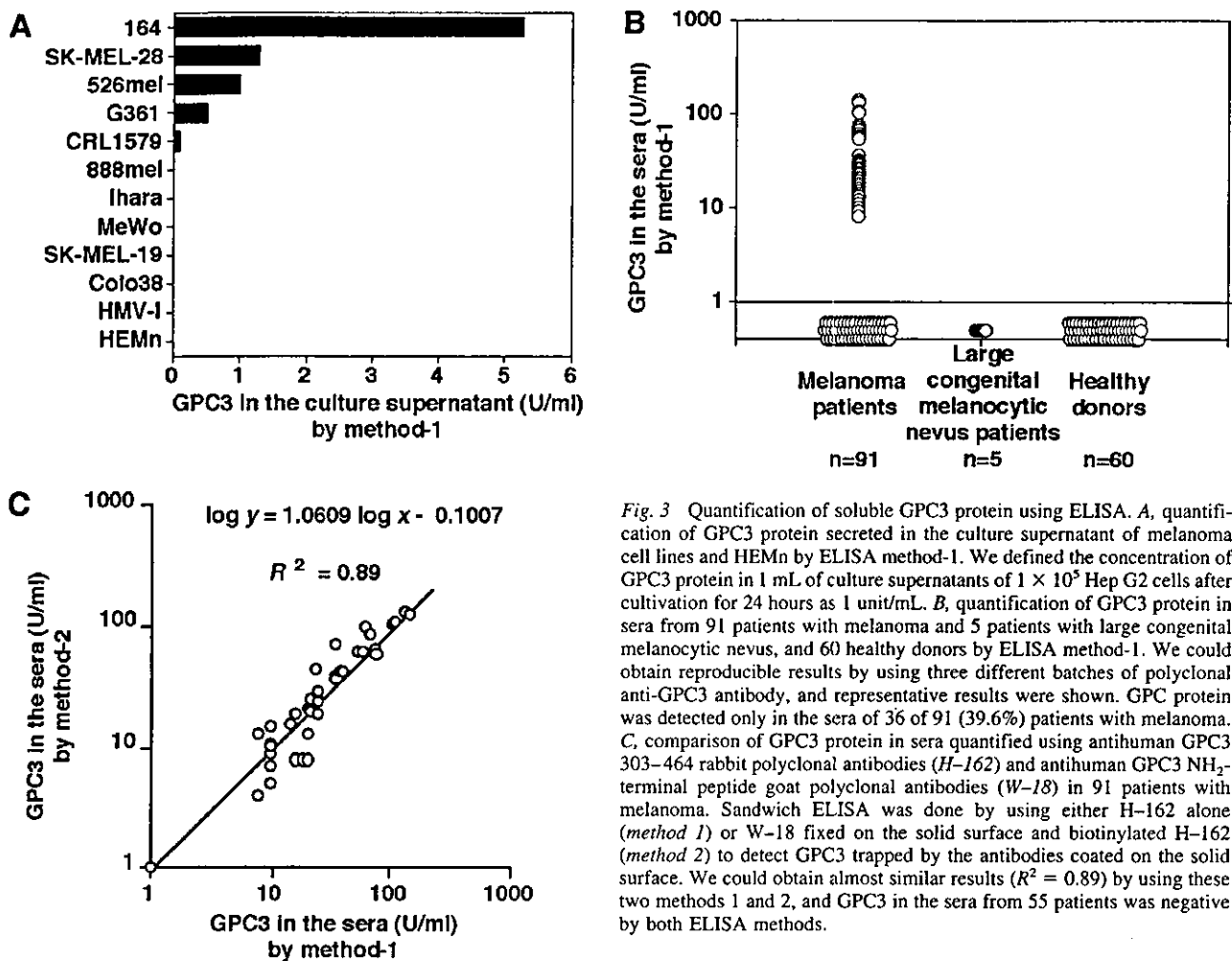
Fig. 3 Quantification of soluble GPC3 protein using ELISA. A, quantification of GPC3 protein secreted in the culture supernatant of melanoma cell lines and HEMn by ELISA method-1. We defined the concentration of GPC3 protein in 1 mL of culture supernatants of 1 × 10⁵ Hep G2 cells after cultivation for 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 NH₂-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 super-

ficial 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
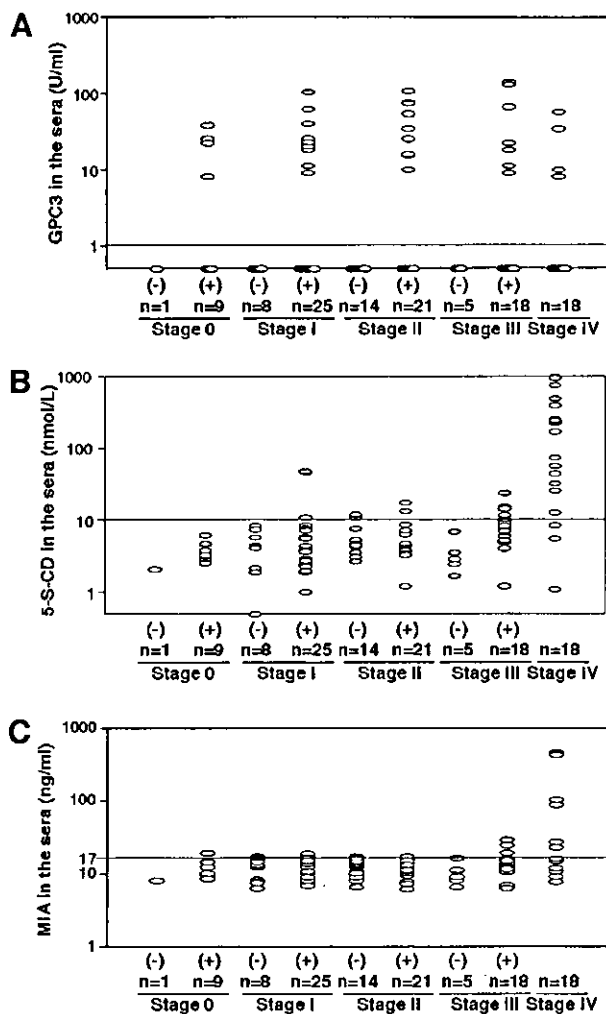
Fig. 4 Comparison of serum concentrations of GPC3, 5-S-CD, and MIA in patients with melanoma classified by stage. *A*, serum concentrations of GPC3 measured by ELISA method-1, (*B*) 5-S-CD, and (*C*) MIA in 91 patients with melanoma (+) and 28 disease-free patients without detectable melanoma (−) classified 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 recur-

rence (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
10	GPC3 (U/ml)†	103‡	17	44	0
	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		
11	GPC3 (U/ml)	40	83		
	5-S-CD (nmol/L)	5.5	NT		
	MIA (ng/ml)	17.2	18.4		

			POD13		
12	GPC3 (U/ml)	25	0		
	5-S-CD (nmol/L)	3.9	NT		
	MIA (ng/ml)	16.5	16.5		

			POD150	POD230	
13	GPC3 (U/ml)	20	0	0	
	5-S-CD (nmol/L)	2.3	2.4	1.8	
	MIA (ng/ml)	6.8	7.5	6.1	

			POD100		
26	GPC3 (U/ml)	61	0		
	5-S-CD (nmol/L)	2.5	6.4		
	MIA (ng/ml)	8.9	12.0		

			POD28		
35	GPC3 (U/ml)	106	0		
	5-S-CD (nmol/L)	17.4	5.0		
	MIA (ng/ml)	10.5	11.1		

			POD124	POD240	
41	GPC3 (U/ml)	75	0	0	
	5-S-CD (nmol/L)	6.4	4.0	4.6	
	MIA (ng/ml)	15.3	16.0	13.6	

			POD4	POD160	
42	GPC3 (U/ml)	73	0	0	
	5-S-CD (nmol/L)	7.0	5.3	7.0	
	MIA (ng/ml)	13.1	9.0	9.2	

			POD32	POD1712	
46	GPC3 (U/ml)	15	0	0	
	5-S-CD (nmol/L)	4.6	6.6	4.5	
	MIA (ng/ml)	9.9	13.0	10.8	

			POD925		
55	GPC3 (U/ml)	10	0		
	5-S-CD (nmol/L)	13.3	11.0		
	MIA (ng/ml)	11.3	14.0		

			POD27	POD50	
68	GPC3 (U/ml)	132	0	0	
	5-S-CD (nmol/L)	8.4	NT	NT	
	MIA (ng/ml)	6.3	14.4	7.6	

			POD98		
69	GPC3 (U/ml)	18	0		
	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. 3A; 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. 4A). 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 downregulated 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.

ACKNOWLEDGMENTS

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Featured Article

Proliferation Potential-Related Protein, an Ideal Esophageal Cancer Antigen for Immunotherapy, Identified Using Complementary DNA Microarray Analysis

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ABSTRACT

Purpose: To establish effective antitumor immunotherapy for esophageal cancer, we tried to identify an useful target antigen of esophageal cancer.

Experimental Design: We did cDNA microarray analysis to find a novel candidate antigen, proliferation potential-related protein (PP-RP). We examined cytotoxicity against tumor cells in vitro and in vivo of CTLs specific to PP-RP established from esophageal cancer patients.

Results: In 26 esophageal cancer tissues, an average of relative ratio of the expression of the *PP-RP* mRNA in cancer cells versus adjacent normal esophageal tissues was 396.2. Immunohistochemical analysis revealed that, in 20 of the 22 esophageal cancer tissues, PP-RP protein was strongly expressed only in the cancer cells and not so in normal esophageal epithelial cells. PP-RP protein contains 10 epitopes recognized by HLA-A24–restricted CTLs. These CTLs, generated from HLA-A24–positive esophageal can-

cer patients, had cytotoxic activity against cancer cell lines positive for both PP-RP and HLA-A24. Furthermore, adoptive transfer of the PP-RP–specific CTL line inhibited the growth of a human esophageal cancer cell line engrafted in nude mice.

Conclusions: The expression of PP-RP in esophageal cancer cells was significantly higher than in normal cells, and the CTLs recognizing PP-RP killed tumor cells in vitro and also showed tumor rejection effects in a xenograft model. Therefore, PP-RP may prove to be an ideal tumor antigen useful for diagnosis and immunotherapy for patients with esophageal cancer. cDNA microarray analysis is a useful method to identify ideal tumor-associated antigens.

INTRODUCTION

Cancer in the esophagus is a worldwide malignant neoplasm in particular in Pacific countries. Surgery remains the standard approach for treatment of patients with locoregional advanced disease that is resectable. Curative resection is feasible in 50% of cases, yet local or distant lesions are common after resection (1). The 5-year survival is only ~30% for stage III and stage IV patients undergoing surgery. Some adjuvant multimodality therapies have been attempted to control both local and systemic disease (2, 3). However, unresectable and relapsed esophageal cancers can be resistant to presently available chemotherapy or radiation therapy regimens, and there is almost no clear advantage of these regimens on overall survival. Consequently, development of a new effective therapeutic approach such as immunotherapy is needed to expand treatment modalities (4, 5). Recently, there have been reports on the clinical efficacy of immunotherapy for advanced cancer in the digestive tract, but little clinical data have been reported in cases of advanced esophageal cancer (6, 7). The presence of precursors of HLA class I-restricted and cancer-specific CTLs in both peripheral blood mononuclear cells (PBMCs) and tumor infiltrating lymphocytes of patients with esophageal cancer have been reported (8, 9).

Many tumor-associated antigens (TAAs) in certain human malignancies were identified using methods of cDNA expression cloning (10–12) and the serological analysis of recombinant cDNA expression library (SEREX) (13–15). These methods promoted renewed efforts to develop antigen-specific immunotherapy, but clinical effects have progressed slowly. To circumvent these obstacles, we initiated studies to search for TAAs that could trigger CTL responses against cancer cells. Rather than analyzing tumor-derived T-cell clones or tumor-specific antibodies derived from patients, we used cDNA microarray analysis to identify TAA genes strongly expressed in tumor cells. Advantages of use of cDNA microarray analysis for

screening of TAAs are as follows: (a) in cDNA microarray analysis used for this study, expression levels of 9216 kinds of genes, approximately one third of all human genes, can be comprehensively investigated in both cancer cells and adjacent normal cells; (b) we can choose TAA with a commonality by investigating mRNA from many patients (26 esophageal cancer patients in this study); and (c) furthermore, we can choose antigens strongly and specifically expressed only in cancer cells and not in normal cell counterparts. Characteristics of TAAs thus selected prevent development of autoimmune diseases after immunotherapy with TAAs.

In the present study, we identified a candidate of an ideal TAA, proliferation potential-related protein (PP-RP), using cDNA microarray analysis of esophageal cancer (16), and generated PP-RP–specific CTL lines. Furthermore, PP-RP appeared to have a role in promoting proliferation of esophageal cancer cells; hence, loss of PP-RP is difficult to occur in esophageal cancer cells. Comprehensively, PP-RP may prove to be an ideal tumor rejection antigen suitable for immunotherapy of esophageal cancer.

MATERIALS AND METHODS

cDNA Microarray Analysis. Profiling of gene expression by cDNA microarray analysis was done, as described previously (16). We obtained primary esophageal cancer and adjacent noncancerous normal esophageal tissues from 26 Japanese patients with esophageal cancer during routine diagnostic procedures after obtaining a formal agreement signed by the patients.

Cell Lines and HLA Expression. Esophageal cancer cell lines, TE1, TE2, TE3, TE8, TE9, TE10, TE11, TE13, and TE14, were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan), and a liver cancer cell line SK-Hep-1 was provided by Dr. Kyogo Itoh of Kurume University (Kurume, Japan). The expression of HLA-A24 was examined using flow cytometry with an anti-HLA-A24 monoclonal antibody (One Lambda, Inc., Canoga Park, CA) to select target cell lines for CTL assays. Human B lymphoblastoid cell line C1R (expressing a trace amount of HLA class I molecule other than HLA-A24) and C1R-A*2404 (C1R cells transfected with an *HLA-A*2402* gene; ref. 17) were generous gifts of Dr. Masafumi Takiguchi (Kumamoto University, Kumamoto, Japan) and used for target cells of cytotoxicity assay after being pulsed with peptides. The origins and HLA genotypes of these cell lines have been described elsewhere (18).

Northern Blot Analysis and Reverse Transcription-PCR (RT-PCR). Northern blot analysis was done as described previously (19). Integrity of RNA in formalin-Mops gels was checked using electrophoresis. Gels with 20 μg of total RNA per lane were blotted onto nylon membrane (Hybond N$^+$; Amersham, Piscataway, NJ). Poly(A)$^+$ RNA blots of human tissues (Human 12-Lane MTN Mlot; Clontech, Palo Alto, CA) was also used. Membranes were hybridized with *PP-RP*–specific cDNA probe (367 to 1307 bp) labeled with [^{32}P]dCTP. RT-PCR analysis of cancer cell lines was done as described previously (20). *PP-RP* gene-specific PCR primer sequences were as follows: sense, 5′-TGCTGTTGTGATTCCCTGCTG-

3′, and antisense, 5′-AGGAAACTGAGGAGAAAACTG-3′, and 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.

Immunohistochemical Staining of Tissue Sections and Immunocytochemical Analysis. Immunohistochemical examinations were done, as described previously (15, 21). The primary antibody used in this study, goat polyclonal anti-RBQ-1 antibody raised against a peptide mapped at the NH$_2$ terminus of human PP-RP origin, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). We stained 4-μm thick sections of formalin-fixed, paraffin-embedded tissue samples with anti-RBQ-1 antibody at a 1:200 dilution. To investigate intracellular localization of PP-RP, cells grown on a 35-mm Petri dish were fixed with cold 100% methanol for 10 minutes and washed twice with washing buffer [80 mmol/L PIPES (pH 6.8), 5 mmol/L EGTA, 1 mmol/L MgCl$_2$, and 0.5% Triton X-100]. The fixed cells were stained with primary antibody, at a 1:200 dilution overnight at 4°C. After washing, cells were incubated with fluorescein-conjugated F(ab′) donkey antigoat IgG (H+L) antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a 1:1000 dilution for 1 hour at room temperature. For detection of nuclear DNA, cells were stained with 0.25 μg/mL propidium iodide for 10 minutes, mounted with glycerol, and observed using confocal microscopy (Fluoview FV300; Olympus, Tokyo, Japan).

Generation of PP-RP Peptide-specific CTL Lines and Cytotoxicity Assay. Peptides (purity > 90%) carrying the sequence of PP-RP with binding motifs for HLA-A*2402–encoded molecules, including tyrosine or phenylalanine at position 2 and isoleucine, leucine, or phenylalanine at positions 9 or 10, were searched for using BioInformatics and Molecular Analysis Section software (Center for Information Technology, NIH, Bethesda, MD), and we synthesized the 10 kinds of peptides (Table 2) using the 9-fluorenyl-methyloxycarbonyl method on an automatic peptide synthesizer (PSSM8; Shimadzu, Kyoto, Japan), then purified using high-performance liquid chromatography. A HIV nef-derived peptide (RYPLTF-GWCF), which can bind to HLA-A*2402–encoded molecules, was used as a negative control (22). We isolated PBMCs from heparinized blood of HLA-A24$^+$ Japanese patients with esophageal cancer and healthy donors by Ficoll-Conray density gradient centrifugation, and dendritic cells were generated as described previously (23, 24). Blood was obtained during routine diagnostic procedures with a formal agreement signed by the donors. The generated cells expressed on their cell surfaces dendritic cell-associated molecules such as CD1a, CD83, CD86, and HLA-DR (data not shown). CD8$^+$ T cells were isolated using CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from PBMCs of the same donors.

CD8$^+$ T cells (2 × 10^6) were cultured with irradiated (3500 cGy) autologous dendritic cells (2 × 10^5) in RPMI 1640 supplemented with 10% heat-inactivated autologous plasma, 5 ng/mL recombinant human interleukin-7 (Pepro Tech EC Ltd., London, United Kingdom), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine together with a 1 μg/mL PP-RP synthetic peptide in a 16-mm well plate. After culture for 7 days, half of the medium was exchanged with fresh

culture medium supplemented with interleukin 7 and 10 units/mL recombinant human interleukin 2, and the cells were again stimulated by adding irradiated (3500 cGy) autologous dendritic cells (2 × 10^5) and 1 µg/mL PP-RP peptide, then the cells were stimulated for a third time on day 7 of the culture. After culture for an additional 6 days on day 20 from the start of culture, the cytotoxicity of growing cells was examined. The CTL lines were cultured continuously in interleukin 2-containing culture medium and irradiated autologous PBMCs, and PP-RP peptides were added to the wells every week. Cytotoxic activity of CTLs was investigated using chromium release assay, as described previously (13).

An Adoptive Immunotherapy Model. BALB/c-nu/nu female mice at 6 weeks of age purchased from Charles River Japan (Yokohama, Japan) and maintained at the Center for Animal Resources and Development of Kumamoto University were handled in accordance with the animal care policy of Kumamoto University. An experimental adoptive immunotherapy was done as described previously (25). For xenografting, TE11 human esophageal cancer cells (1 × 10^5) were injected s.c. into the right flank of each mouse. When tumor size became 30 mm^2, a PP-RP peptide-8–specific CTL line or control CD8^+ T-cell line (5 × 10^6) established from patient 5 suspended in 100 µL of PBS was injected into the tumor. The control CD8^+ T-cell line was established from CD8^+ T cells of patient 5 by stimulation with PP-RP peptide-5 and exhibited neither PP-RP peptide-5–specific cytotoxic activity nor cytotoxicity against TE11 cells *in vitro*. Each CTL-treated and control groups included five mice. On days 0 and 7, the mice received injection of an additional dose of T cells (5 × 10^6) or PBS alone, and the groups were monitored for tumor growth until all of the mice in the control group died. Sizes of the tumors were measured at 3-day intervals, and tumor volumes were calculated using the ellipsoid formula (length × width). Two-tailed Student's *t* test was used to determine the statistical significance of differences in tumor growth between the CTL-treated and control groups. A value of $P < 0.05$ was considered significant. The Kaplan-Meier plot for survivals was assessed for significance of difference in mice survivals between two groups using the Breslow-Gehan-Wilcoxon test.

Generation of a PP-RP Stable Knockdown Cell and Cell Proliferation Assay. We designed short hairpin RNA, as previously described (26, 27), referring to technical information of Ambion, Inc. (Austin, TX), and NipponBioService (Asaka, Saitama, Japan). We used the pSilencer vector (Ambion, Inc.), a stable expression vector of short hairpin RNA, to interfere with *PP-RP* gene expression. The targeted small interfering RNA sequences corresponded to bases 326–345 of the *PP-RP* gene: short hairpin RNA oligo-sense, 5'-GATCCCGAACAG-CACTCCTGGAATCTTCAAGAGAGATTCCAGGAGTG-CTGTTCTTTTTTGGAAA-3', and short hairpin RNA oligo-antisense, 5'-AGCTTTTCCAAAAAAGAACAGCACTCC-TGGAATCTCTCTTGAAGATTCCAGGAGTGCTGTTC-GG-3'. The TE13 cell was transfected with the pSilencer-PP-RP or pSilencer-GFP (mock) construct containing a hygromycin-resistance marker by LipofectAMINE 2000 Reagent (Invitrogen Corp., Carlsbad, CA). Cells were selected with 500 µg/mL hygromycin, and resistant cells were propagated. To investigate

the effect of suppression of PP-RP gene expression on proliferation of cancer cells, hygromycin-resistant cells, obtained after transfection, were plated at a density of 1 × 10^5 cells in a 60-mm tissue culture dish and cultured in RPMI 1640 supplemented with 5 or 1% FCS. Cell numbers were counted for consecutive 10 days.
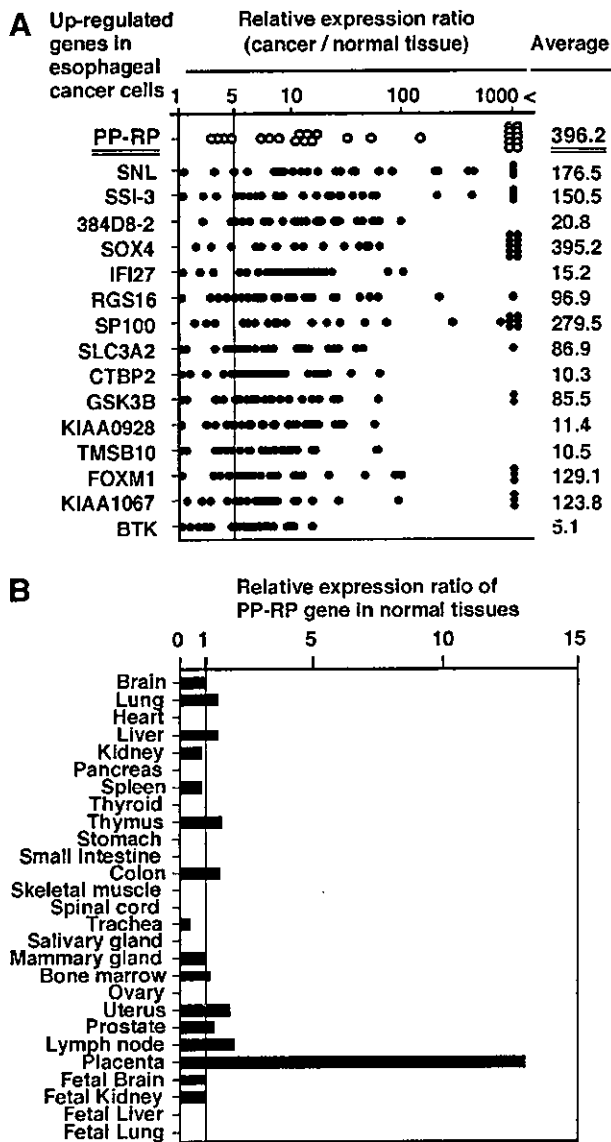
RESULTS

Markedly Enhanced Expression of *PP-RP* mRNA in Esophageal Cancer Tissue and Cell Lines. Our data comparing the relative expression ratio of 9216 kinds of genes among 26 cases of esophageal cancer tissues and their adjacent normal counterparts, using cDNA microarray analysis, were reported previously (16). After analysis, we chose 16 genes of which the relative expression ratio was more than five times higher in >20 of 26 patients with esophageal cancer (Fig. 1A). Thereby, we analyzed the expression of these genes using cDNA microarray analysis in 27 kinds (including 4 embryonic tissues) of normal tissues (Fig. 1B). Consequently we identified PP-RP to be an ideal target for immunotherapies for esophageal cancer patients. In 22 of 26 patients, the expression of *PP-RP* gene in cancer cells was more than five times higher than that in normal counterparts (average of relative expression ratio: 396.2; Fig. 1A). In addition, *PP-RP* gene was strongly expressed only in the placenta, as based on cDNA microarray analysis (Fig. 1B).

Expression of the *PP-RP* gene in esophageal cancer cell lines and normal tissues at the mRNA level was also analyzed using RT-PCR and Northern blot analysis. TE1, TE2, TE3, TE8, TE9, TE10, TE11, TE13, and TE14 esophageal cancer cell lines and three esophageal cancer tissues strongly expressed *PP-RP* mRNA, although a liver cancer cell line SK-Hep-1 did not (Fig. 2A). *PP-RP* mRNA was strongly expressed in the normal placenta and testis, but not so in the normal esophagus, and was weakly expressed in brain, heart, kidney, lung, and liver (Fig. 2B).

Expression of PP-RP Protein Only in Placenta, Testis, and Esophageal Cancer Cells. To investigate the expression of PP-RP protein, we then examined many paraffin-embedded normal tissues and 22 cases of paraffin-embedded esophageal cancer tissues. PP-RP was stained in normal testis and placenta (Fig. 3A–D) but not so in the normal spleen, lymph node, brain, kidney, lung, and liver (Fig. 3E–P). In the testis, the germ cells were diffusely stained in the nucleus and cytoplasm, using anti-PP-RP antibodies. Trophoblasts were mainly stained in the placenta. In almost all esophageal cancer tissues tested, PP-RP staining was observed in the cancer cells, whereas in contrast, no significant PP-RP staining was observed in epithelial cells in the normal esophagus (Fig. 3Q–X). The staining of PP-RP protein colocalized with chromosomes in mitotic cells, as seen under higher magnification fields (Fig. 3, W and X).

Higher *PP-RP* Expression Was Associated with a Poor Prognosis of Patients with Microscopic Residual Tumor Cells after Surgery. As shown in Fig. 1A, there was a wide range of variation of *PP-RP* expression in cancer tissues derived from 26 patients. On the basis of the pathological analysis of the surgically removed tissues, the patients were classified into three groups: R0 (8 patients with no residual tumor after surgical

A Up-regulated genes in esophageal cancer cells

Relative expression ratio (cancer / normal tissue)

Average

		Average
PP-RP		396.2
SNL		176.5
SSI-3		150.5
384D8-2		20.8
SOX4		395.2
IFI27		15.2
RGS16		96.9
SP100		279.5
SLC3A2		86.9
CTBP2		10.3
GSK3B		85.5
KIAA0928		11.4
TMSB10		10.5
FOXM1		129.1
KIAA1067		123.8
BTK		5.1

B Relative expression ratio of PP-RP gene in normal tissues

Fig. 1 Markedly enhanced expression of *PP-RP* mRNA in esophageal cancer tissues as based on cDNA microarray analysis. *A*, a list of up-regulated genes in esophageal cancer cells (16). These genes were expressed more than five times higher in cancer cells as compared with normal counterparts in >20 of 26 esophageal cancer patients. We selected *PP-RP* among these genes as a candidate antigen for esophageal cancer immunotherapy. The expression of *PP-RP* mRNA in esophageal cancer cells was markedly enhanced in 22 of 26 esophageal cancer patients. *B*. In normal tissues, the *PP-RP* gene was strongly expressed only in placenta based on cDNA microarray analysis.

resection); R1 (15 patients with microscopic residual tumor); and R2 (3 patients with macroscopic residual tumor). Group R1 was additionally divided into two subgroups based on the microarray analysis: R1A (5 patients with a relative *PP-RP* expression ratio > 1000) and R1B (10 patients with a relative *PP-RP* expression ratio of 2–142). As shown in Table 1 and Fig. 4, all of the five patients of R1A group, with a very high *PP-RP* expression in cancer cells, died within 24 months. In contrast, 7 of 10 patients of group R1B survived for >30–122 months

(Table 1 and Fig. 4). The difference in patient survival between the two subgroups is statistically significant (P < 0.05, Breslow-Gehan-Wilcoxon test).

Establishment of CTL Lines Specific to PP-RP–derived Peptides and Cytotoxicity of CTL Lines against Cancer Cell Lines. We tried to generate PP-RP–specific CTL lines from PBMCs of five patients with esophageal cancer and six healthy donors positive for HLA-A24 using stimulation by dendritic cells pulsed with PP-RP–derived peptide and established CTL lines, which had cytotoxic activity to a PP-RP peptide-loaded, but not unloaded, C1R-A*2402 cells. Peptide specificity of representative four CTL lines, as shown in Fig. 5, exhibited cytotoxic activity to C1R-A*2402 cells loaded with the PP-RP peptides but not to a unloaded or PP-RP peptide-loaded parent cell line, C1R cells, negative for *HLA-A*2402*. These CTL lines also did not exhibit cytotoxic activity to C1R-A*2402 cells loaded with an irrelevant HIV-1 nef-derived peptide having high binding affinity to HLA-A24 (Fig. 5A–D). These data indicated that the cytotoxic activity of these CTL lines was PP-RP derived peptide specific and was restricted by HLA-A24.

Subsequently, we asked whether these CTL lines could lyse esophageal cancer cells expressing PP-RP. HLA-A24 expression in the cancer cell lines was examined by staining with anti-HLA-A24 monoclonal antibody (One Lambda, Inc., Canoga Park, CA), followed by flow cytometry (data not shown) and genotyping. Expression of PP-RP was examined using RT-PCR (Fig. 2A) and immunocytochemical analysis (Fig. 9C).
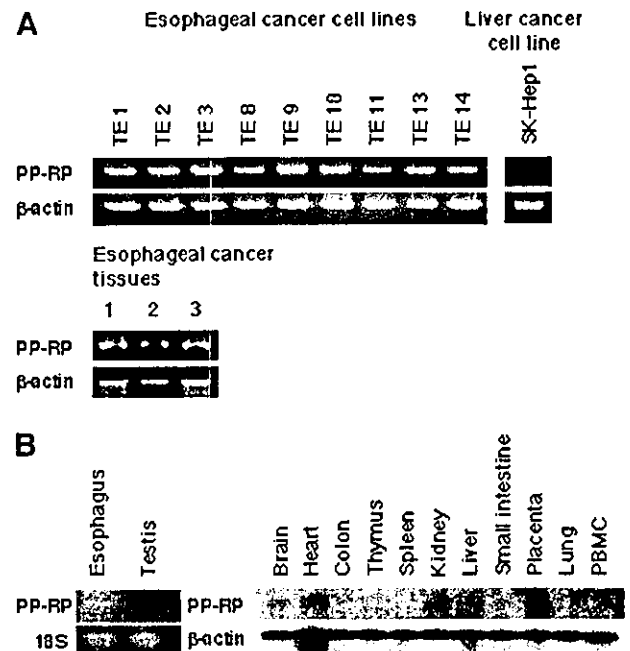
Fig. 2 Expression of *PP-RP* mRNA in cancer cell lines, cancer tissues, and normal tissues. *A*, RT-PCR analysis of *PP-RP* expression in various cancer cell lines and three esophageal cancer tissues. The same cDNA samples were tested for β-*actin* expression as a control. RT-PCR was done in at least two independent and reproducible experiments. *B*, Northern blot analysis of *PP-RP* mRNA in various normal tissues. The same filter was stripped and rehybridized with a β-*actin* probe to prove RNA integrity and to assess loading of equal amounts of RNA.
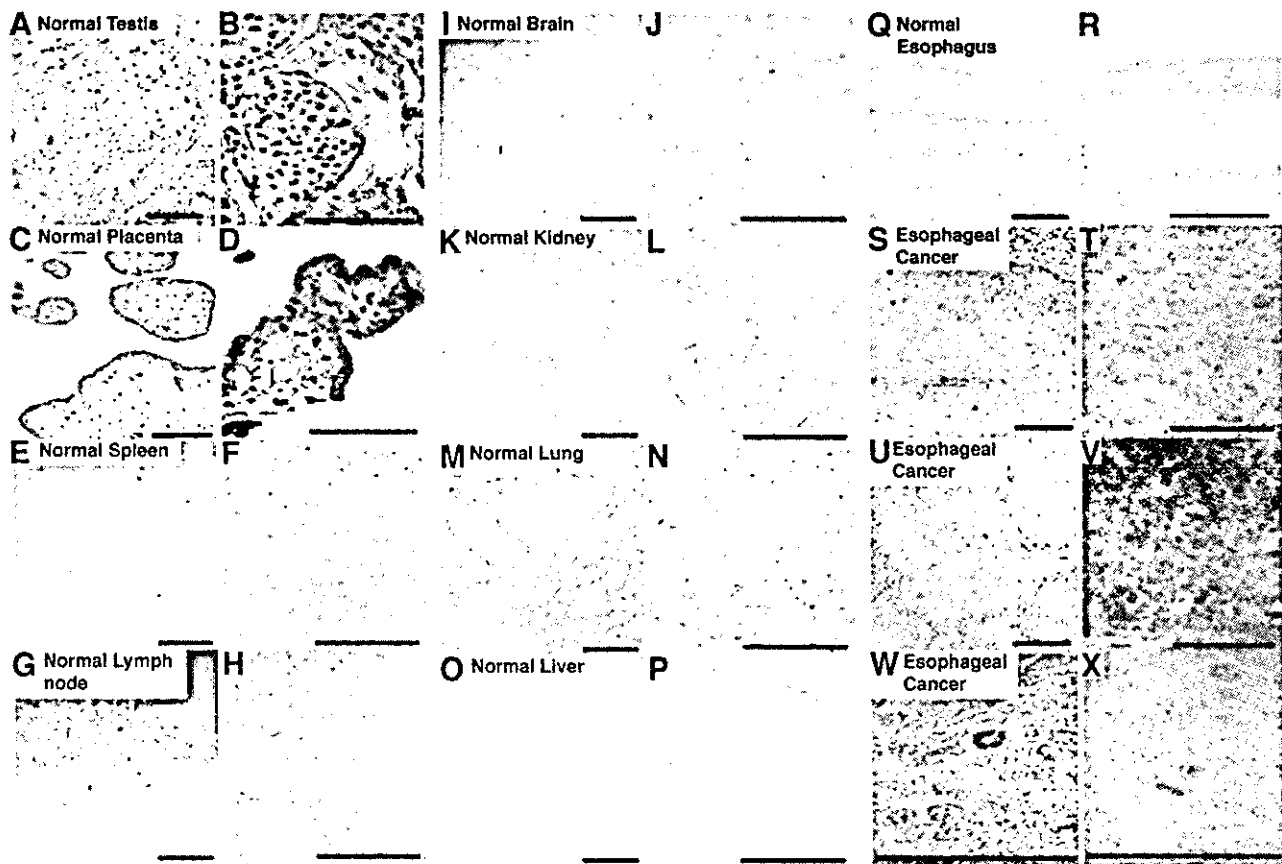
Fig. 3 Immunohistochemical staining of PP-RP protein. Normal testis (*A* and *B*), normal placenta (*C* and *D*), normal spleen (*E* and *F*), normal lymph node (*G* and *H*), normal brain (*I* and *J*), normal kidney (*K* and *L*), normal lung (*M* and *N*), normal liver (*O* and *P*), normal esophagus (*Q* and *R*), and esophageal cancer tissues (*S-X*) were analyzed. Sections were stained with anti-PP-RP antibody and developed using an avidin-biotin complex method with 3,3′-diaminobenzidine. Positive staining signals are seen as *brown. Scale bars* represent 100 μm.

Table 1 Clinicopathological features of 15 cases of R1 esophageal cancer patients

Gender	Age (y)	Relative expression ratio of *PP-RP* gene in cancer tissue	Outcome*	Survival period (mo)†	Tumor-node-metastasis stage	Histopathological grading‡	Residual tumor§
M	43	>1000	Deceased	24	IV	G2	R1
M	60	>1000	Deceased	16	IV	G2	R1
M	55	>1000	Deceased	16	IV	G1	R1
M	56	>1000	Deceased	12	IV	G2	R1
M	60	>1000	Deceased	4	IV	G1	R1
M	61	142.1	Survived	>62	IV	G1	R1
M	61	48.6	Survived	>98	IV	G2	R1
M	54	11	Survived	>122	III	G2	R1
M	50	10.5	Survived	>34	IV	G1	R1
M	58	10.2	Deceased	19	IV	G2	R1
M	67	6.3	Survived	>30	IV	G2	R1
M	67	6	Survived	>30	IV	G2	R1
M	63	5	Deceased	11	IV	G2	R1
M	57	2.2	Survived	>30	IV	G2	R1
M	55	2.1	Deceased	20	IV	G2	R1

* Eight patients died from causes related to esophageal cancer.
† Follow-up months were established as the time between surgery and either death or the last follow-up date.
‡ G1, G2, and G3 represent well, moderately, or poorly differentiated squamous cell carcinoma, respectively.
§ R1 represents neither R0 nor R2; R0, no residual tumor; R2, macroscopic residual tumor.
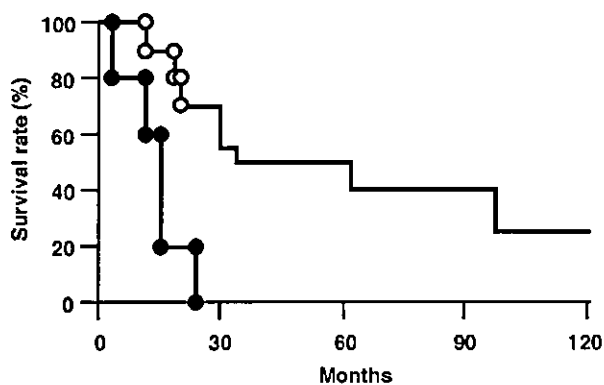
Fig. 4 Association between higher expression levels of the *PP-RP* gene and poor prognoses after surgery in 15 R1 esophageal cancer patients. All patients of R1A group, with very high *PP-RP* expression in cancer cells, died within 24 months (●). In contrast, 7 of 10 patients of R1B group (relative *PP-RP* expression ratio of 2 to 142) survived for >30 to 122 months (○). The prolonged survival of R1B group was statistically significant ($P < 0.05$).
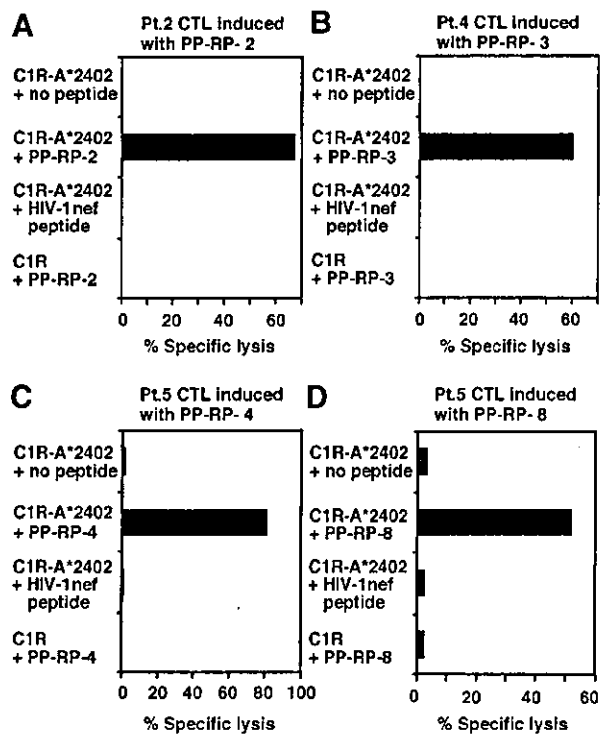

Fig. 5 PP-RP–derived peptide-specific and HLA-A*2402–restricted cytotoxicity of CTL lines. CTL lines specific to PP-RP-2 (*A*), PP-RP-3 (*B*), PP-RP-4 (*C*), or PP-RP-8 (*D*) were generated from esophageal cancer patients 2, 4, 5, or 5, respectively. Cytotoxic activities of CTL lines against C1R or A*2402-transfected C1R cells prepulsed with indicated peptides were detected in a 4-hour ^{51}Cr release assay at E:T ratio of 20.

TE11, TE13, and SK-Hep1 expressed HLA-A24, whereas TE9 expressed HLA-A33 but not HLA-A24. TE11, TE13, and TE9 but not SK-Hep1 expressed PP-RP at high levels. Representative data of the cytotoxicity of four kinds of peptide-specific

CTL lines against TE11 and other cancer cell lines are shown in Fig. 6. These CTL lines exhibited cytotoxicity only against TE11 and TE13 positive for both HLA-A24 and PP-RP but not against TE9 and SK-Hep1 (Fig. 6*A*–*D*).

All data of induced CTL lines concerning PP-RP–derived peptide specificity and cytotoxicity against cancer cells expressing both HLA-A24 and PP-RP and inducible rates for such CTL lines for each peptide or each patient are summarized in Table 2. These results indicate that all 10 PP-RP–derived peptides have the capacity to induce such CTL lines, and these CTL lines were induced from all five esophageal cancer patients but not from six healthy donors.

To additionally confirm that the cytotoxicity of these CTL lines against cancer cells was mediated by specific recognition of endogenously processed PP-RP, we generated PP-RP stable knockdown TE13 cells by transfection with a pSilencer vector containing human PP-RP small interfering RNA, TE13shPP-RP. As control cells, we also generated TE13 cells transfected with a pSilencer vector containing green fluorescent protein (GFP) small interfering RNA, TE13shGFP. Cells were selected with hygromycin, and resistant cells were cultured. After 2 months of culture, PP-RP protein showed a significant reduction in TE13shPP-RP cells. In Western blot analysis, PP-RP was much lower in TE13shPP-RP cells than in TE13 cells and TE13shGFP cells (Fig. 7*A*). We then did a ^{51}Cr release assay using these cells as target. The CTL lines exhibited cytotoxicity against TE13 cells and TE13shGFP cells but not against
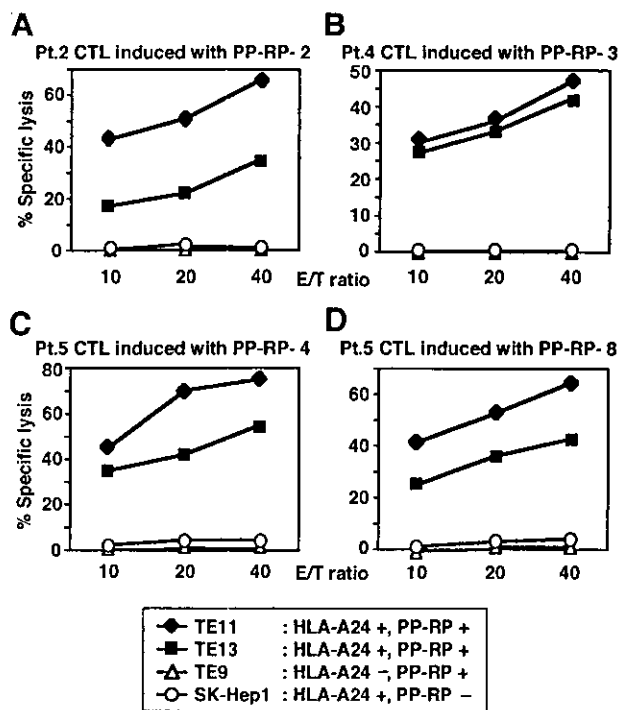
Fig. 6 Cytotoxic activities of peptide-induced CTL lines against cancer cell lines. CTL lines used were the same as those shown in Fig. 5. Cytotoxic activities of CTL lines against cancer cell lines were detected in a 4-hour ^{51}Cr release assay at the indicated E:T ratios. An assay was set up in duplicate wells, and mean values were used for calculation of percent-specific lysis.