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

protein S100 enable early detection of melanoma recurrence? Melanoma Res 2000;10:451–9.

25. Reinhardt MJ, Kensy J, Frohmann JP, et al. Value of tumour marker S-100B in melanoma patients: a comparison to 18F-FDG PET and clinical data. Nuklearmedizin 2002;41:143–7.

26. Hanawa Y, Wakamatsu K, Ikeda S. Serum concentration of 5-S-cysteinyldopa in pediatric patients with giant pigmented nevi. J Dermatol 1996;23:16–21.

27. Capurro M, Wanless IR, Sherman M, et al. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 2003;125:89–97.

28. Hippo Y, Watanabe K, Watanabe A, et al. Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res 2004;64:2418–23.

29. Dong J, Phelps RG, Qiao R, et al. BRAF oncogenic mutations correlate with progression rather than initiation of human melanoma. Cancer Res 2003;63:3883–5.

30. Pilia G, Hughes-Benzie RM, MacKenzie A, et al. Mutations in GPC3, a glypican gene, cause the Simpson-Golabi-Behmel overgrowth syndrome. Nat Genet 1996;12:241–7.

31. Neri G, Gurrieri F, Zanni G, Lin A. Clinical and molecular aspects of the Simpson-Golabi-Behmel syndrome. Am J Med Genet 1998;79: 279–83.

32. Garganta CL, Bodurtha JN. Report of another family with Simpson-Golabi-Behmel syndrome and a review of the literature. Am J Med Genet 1992;44:129–35.

33. Gurrieri F, Cappa M, Neri G. Further delineation of the Simpson-Golabi-Behmel (SGB) syndrome. Am J Med Genet 1992;44:136–7.

34. Hughes-Benzie RM, Pilia G, Xuan JY, et al. Simpson-Golabi-Behmel syndrome: genotype/phenotype analysis of 18 affected males from 7 unrelated families. Am J Med Genet 1996;66:227–34.

35. Xuan JY, Hughes-Benzie RM, MacKenzie AE. A small interstitial deletion in the GPC3 gene causes Simpson-Golabi-Behmel syndrome in a Dutch-Canadian family. J Med Genet 1999;36:57–8.

36. Cano-Gauci DF, Song HH, Yang H, et al. Glypican-3-deficient mice exhibit developmental overgrowth and some of the abnormalities typical of Simpson-Golabi-Behmel syndrome. J Cell Biol 1999;146: 255–64.

37. Gonzalez AD, Kaya M, Shi W, et al. OCI-5/GPC3, a glypican encoded by a gene that is mutated in the Simpson-Golabi-Behmel overgrowth syndrome, induces apoptosis in a cell line-specific manner. J Cell Biol 1998;141:1407–14.

38. Lin H, Huber R, Schlessinger D, Morin PJ. Frequent silencing of the GPC3 gene in ovarian cancer cell lines. Cancer Res 1999;59: 807–10.

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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REFERENCES

1. Brochez L, Naeyaert JM. Serological markers for melanoma. Br J Dermatol 2000;143:256–68.

2. Hauschild A, Glaser R, Christophers E. Quantification of melanoma-associated molecules in plasma/serum of melanoma patients. Recent Results Cancer Res 2001;158:169–77.

3. Hartleb J, Arndt R. Cysteine and indole derivatives as markers for malignant melanoma. J Chromatogr B Biomed Sci 2001;764:409–43.

4. Wimmer I, Meyer JC, Seifert B, Dummer R, Flace A, Burg G. Prognostic value of serum 5-S-cysteinyldopa for monitoring human metastatic melanoma during immunochemotherapy. Cancer Res 1997; 57:5073–6.

5. Wakamatsu K, Yokochi M, Naito A, Kageshita T, Ito S. Comparison of phaeomelanin and its precursor 5-S-cysteinyldopa in the serum of melanoma patients. Melanoma Res 2003;13:357–63.

6. Wakamatsu K, Kageshita T, Furue M, et al. Evaluation of 5-S-cysteinyldopa as a marker of melanoma progression: 10 years' experience. Melanoma Res 2002;12:245–53.

7. Hirai S, Kageshita T, Kimura T, et al. Serum levels of sICAM-1 and 5-S-cysteinyldopa as markers of melanoma progression. Melanoma Res 1997;7:58–62.

8. Bosserhoff AK, Kaufmann M, Kaluza B, et al. Melanoma-inhibiting activity, a novel serum marker for progression of malignant melanoma. Cancer Res 1997;57:3149–53.

9. Muhlbauer M, Langenbach N, Stolz W, et al. Detection of melanoma cells in the blood of melanoma patients by melanoma-inhibitory activity (MIA) reverse transcription-PCR. Clin Cancer Res 1999;5:1099–105.

10. Nakatsura T, Yoshitake Y, Senju S, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 2003;306:16–25.

11. Balch CM, Buzaid AC, Soong SJ, et al. Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. J Clin Oncol 2001;19:3635–48.

12. Nakatsura T, Senju S, Yamada K, Jotsuka T, Ogawa M, Nishimura Y. Gene cloning of immunogenic antigens overexpressed in pancreatic cancer. Biochem Biophys Res Commun 2001;281:936–44.

13. Kai M, Nakatsura T, Egami H, Senju S, Nishimura Y, Ogawa M. Heat shock protein 105 is overexpressed in a variety of human tumors. Oncol Rep 2003;10:1777–82.

14. Kageshita T, Ishihara T, Tokuo H, et al. Widespread expression of parathyroid hormone-related peptide in melanocytic cells. Br J Dermatol 2003;148:533–8.

15. Kageshita T, Nakamura T, Yamada M, Kuriya N, Arao T, Ferrone S. Differential expression of melanoma associated antigens in acral lentiginous melanoma and in nodular melanoma lesions. Cancer Res 1991;51:1726–32.

16. Krementz ET, Feed RJ, Coleman WP, 3rd, Sutherland CM, Carter RD, Campbell M. Acral lentiginous melanoma. A clinicopathologic entity. Ann Surg 1982;195:632–45.

17. Kageshita T, Hamby CV, Hirai S, Kimura T, Ono T, Ferrone S. Differential clinical significance of alpha(v)Beta(3) expression in primary lesions of acral lentiginous melanoma and of other melanoma histotypes. Int J Cancer 2000;89:153–9.

18. Sung YK, Hwang SY, Farooq M, Kim JC, Kim MK. Growth promotion of HepG2 hepatoma cells by antisense-mediated knockdown of glypican-3 is independent of insulin-like growth factor 2 signaling. Exp Mol Med 2003;35:257–62.

19. Guadagni F, Ferroni P, Carlini S, et al. A re-evaluation of carcinoembryonic antigen (CEA) as a serum marker for breast cancer: a prospective longitudinal study. Clin Cancer Res 2001;7:2357–62.

20. Maehara Y, Sugimachi K, Akagi M, Kakegawa T, Shimazu H, Tomita M. Serum carcinoembryonic antigen level increases correlate with tumor progression in patients with differentiated gastric carcinoma following noncurative resection. Cancer Res 1990;50:3952–5.

21. Harmenberg U, Wahren B, Wiechel KL. Tumor markers carbohydrate antigens CA 19–9 and CA-50 and carcinoembryonic antigen in pancreatic cancer and benign diseases of the pancreatobiliary tract. Cancer Res 1988;48:1985–8.

22. Gerl A, Lamerz R, Clemm C, Mann K, Hartenstein R, Wilmanns W. Does serum tumor marker half-life complement pretreatment risk stratification in metastatic nonseminomatous germ cell tumors? Clin Cancer Res 1996;2:1565–70.

23. Kageshita T, Yoshii A, Kimura T, et al. Clinical relevance of ICAM-1 expression in primary lesions and serum of patients with malignant melanoma. Cancer Res 1993;53:4927–32.

24. Schlagenhauff B, Schittek B, Ellwanger U, et al. Significance of serum protein S100 levels in screening for melanoma metastasis: does

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 (RYPLTFGWCF), 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 (Miltenyl 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

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 cancer 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

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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′-GATCCCGAACAGCACTCCTGGAATCTTCAAGAGAGATTCCAGGAGTGCTGTTCTTTTTTGGAAA-3′, and short hairpin RNA oligo-antisense, 5′-AGCTTTTCCAAAAAAGAACAGCACTCCTGGAATCTCTCTTGAAGATTCCAGGAGTGCTGTTCGG-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. 1*A*). Thereby, we analyzed the expression of these genes using cDNA microarray analysis in 27 kinds (including 4 embryonic tissues) of normal tissues (Fig. 1*B*). 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. 1*A*). In addition, *PP-RP* gene was strongly expressed only in the placenta, as based on cDNA microarray analysis (Fig. 1*B*).

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. 2*A*). *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. 2*B*).

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. 3*A–D*) but not so in the normal spleen, lymph node, brain, kidney, lung, and liver (Fig. 3*E–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. 3*Q–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. 1*A*, 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

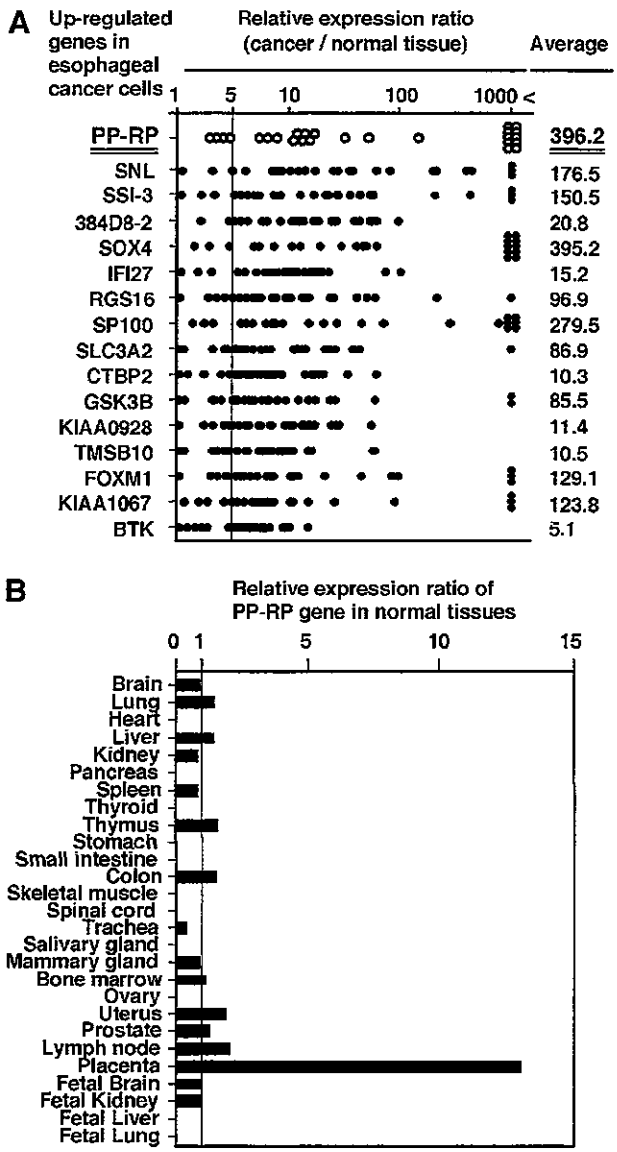

Fig. 1 Markedly enhanced expression of *PP-RP* mRNA in esophageal cancer tissues as based on cDNA microarray analysis. *A*, a list of up-regulated genes in esophageal cancer cells (16). These genes were expressed more than five times higher in cancer cells as compared with normal counterparts in >20 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. 5*A–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. 2*A*) and immunocytochemical analysis (Fig. 9*C*).

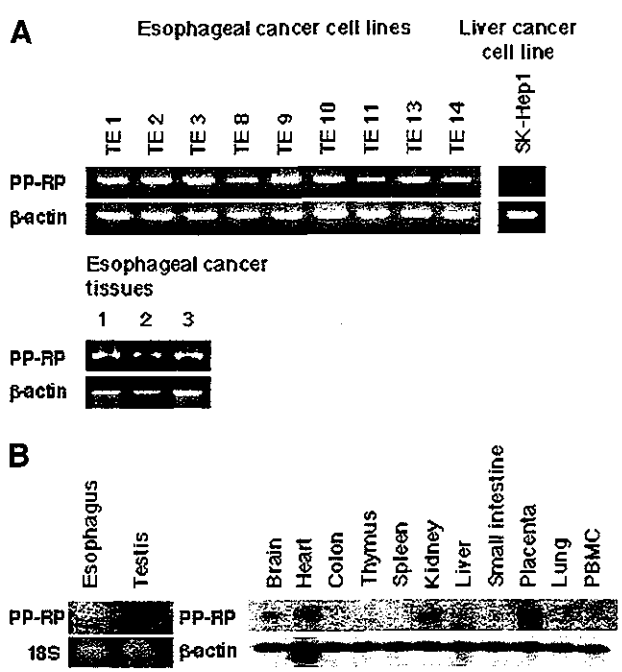

Fig. 2 Expression of *PP-RP* mRNA in cancer cell lines, cancer tissues, and normal tissues. *A*, RT-PCR analysis of *PP-RP* expression in various cancer cell lines and three esophageal cancer tissues. The same cDNA samples were tested for β-*actin* expression as a control. RT-PCR was done in at least 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.

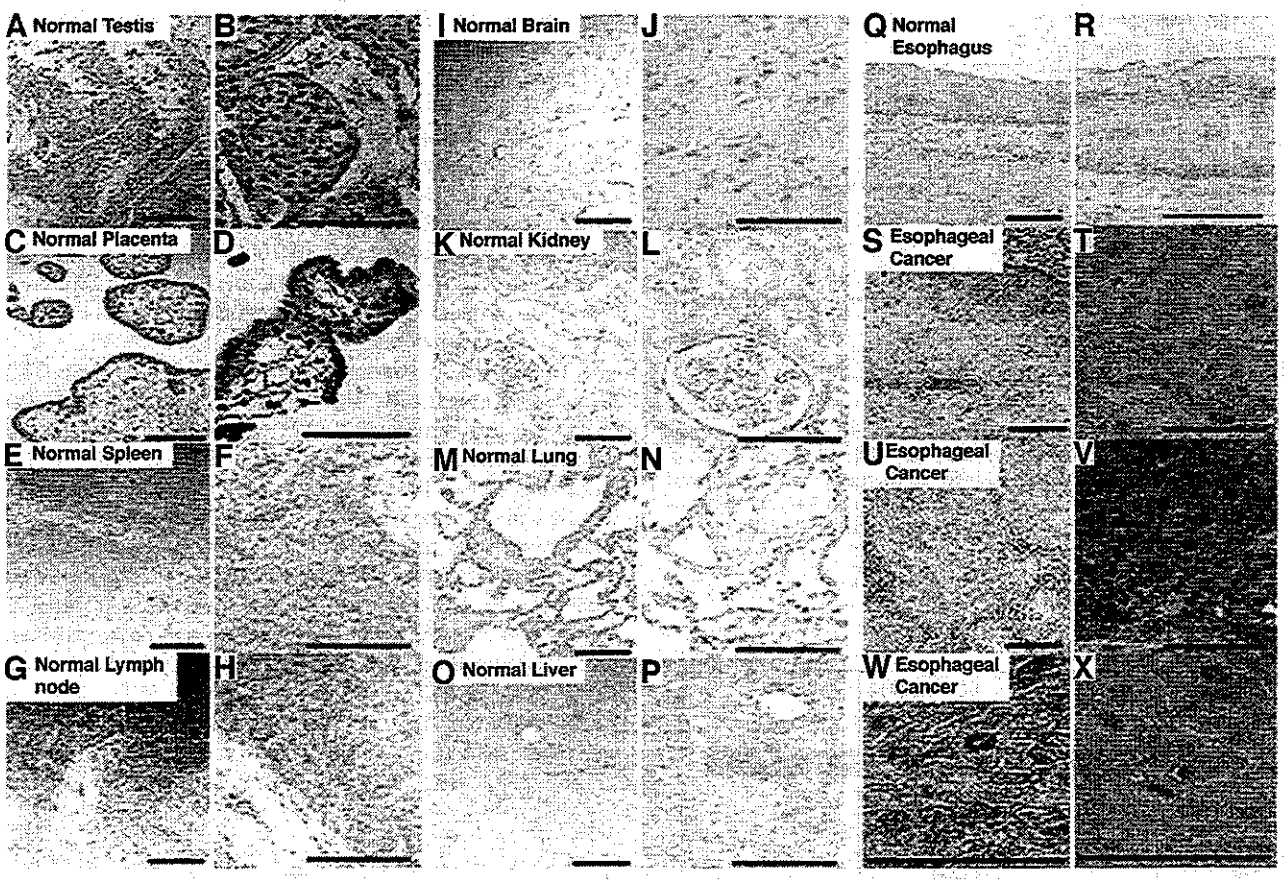

Fig. 3 Immunohistochemical staining of PP-RP protein. Normal testis (*A* and *B*), normal placenta (*C* and *D*), normal spleen (*E* and *F*), normal lymph node (*G* and *H*), normal brain (*I* and *J*), normal kidney (*K* and *L*), normal lung (*M* and *N*), normal liver (*O* and *P*), normal esophagus (*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.

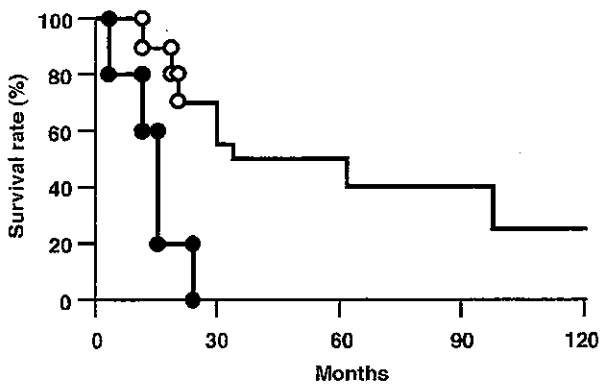

Fig. 4 Association between higher expression levels of the *PP-RP* gene and poor prognoses after surgery in 15 R1 esophageal cancer patients. All patients of R1A group, with very high *PP-RP* expression in cancer cells, died within 24 months (●). In contrast, 7 of 10 patients of R1B group (relative *PP-RP* expression ratio of 2 to 142) survived for >30 to 122 months (○). The prolonged survival of R1B group was statistically significant ($P < 0.05$).

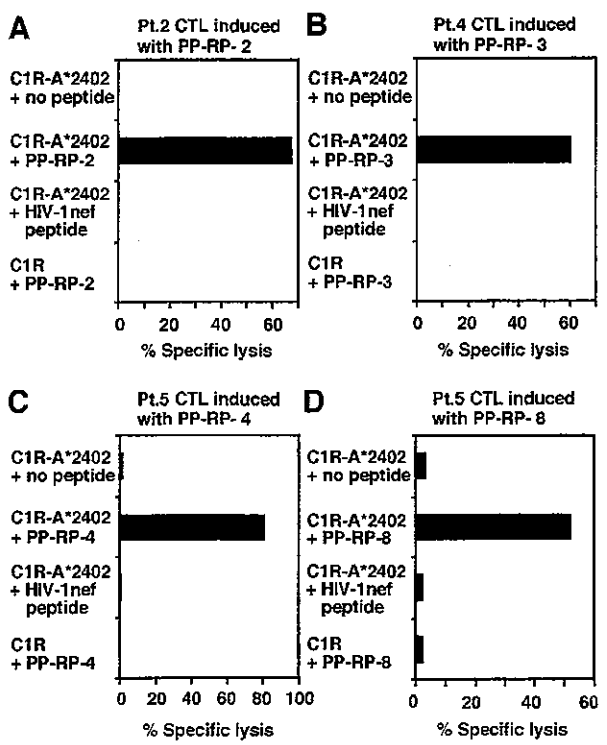

Fig. 5 PP-RP–derived peptide-specific and HLA-A*2402–restricted cytotoxicity of CTL lines. CTL lines specific to PP-RP-2 (*A*), PP-RP-3 (*B*), PP-RP-4 (*C*), or PP-RP-8 (*D*) were generated from esophageal cancer patients 2, 4, 5, or 5, respectively. Cytotoxic activities of CTL lines against C1R or *A*2402*-transfected 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

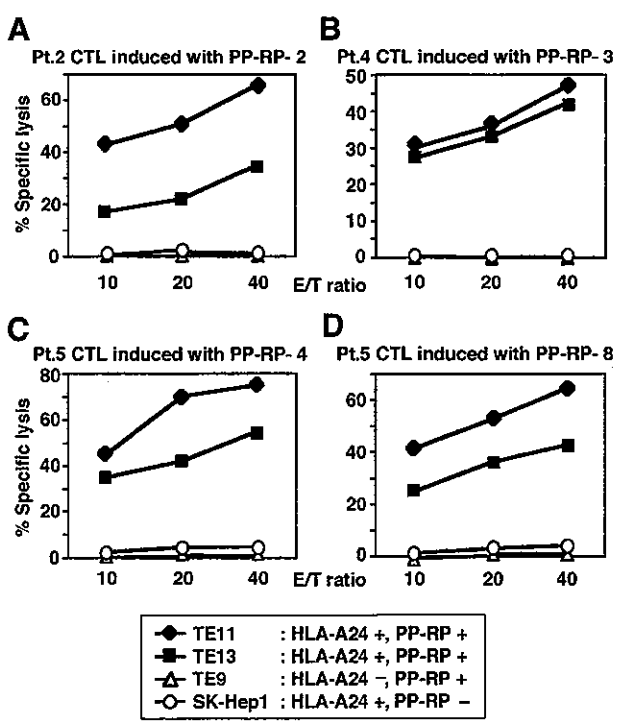

Fig. 6 Cytotoxic activities of peptide-induced CTL lines against cancer cell lines. CTL lines used were the same as those shown in Fig. 5. Cytotoxic activities of CTL lines against cancer cell lines were detected in a 4-hour ^{51}Cr release assay at the indicated E:T ratios. An assay was set up in duplicate wells, and mean values were used for calculation of percent-specific lysis.

Table 2 PP-RP–derived peptides carrying the HLA-A24 binding motif and the inducible rate of CTL lines in HLA-A24$^+$ donors

PP-RP-derived peptides				CTL induction from PBMCs HLA-A24$^+$ patients					CTL-inducible rate for each peptide	CTL induction from PBMCs HLA-A24$^+$ healthy donors						CTL-inducible rate for each peptide
No.	Position	Sequence	Binding score*	1	2	3	4	5		1	2	3	4	5	6	
PP-RP-1	137–146	KFLRQAVNNF	36	−†	−	−	−	+	1/5	−	−	−	−	−	−	0/5
PP-RP-2	151–160	GYTKRLRKQL	240	−	+	+	+	+	4/5	−	−	−	−	−	−	0/5
PP-RP-3	336–345	GYLVSPPQQI	90	−	+	−	+	+	3/5	−	−	−	−	−	−	0/5
PP-RP-4	379–388	VFVPVPPPPL	36	−	−	+	−	+	2/5	−	−	−	−	−	−	0/5
PP-RP-5	388–396	LYPPPPHTL	360	+	−	+	−	−	2/5	−	−	−	−	−	−	0/5
PP-RP-6	420–428	GYSVPPPGF	100	+	−	+	−	−	2/5	−	−	−	−	−	−	0/5
PP-RP-7	464–472	EFYREQRRL	36	+	−	−	+	−	2/5	−	−	−	−	−	−	0/5
PP-RP-8	484–493	EFTNDFAKEL	22	+	−	+	−	+	3/5	−	−	−	−	−	−	0/5
PP-RP-9	622–630	RYREVPPPY	20	+	−	+	+	+	4/5	−	−	−	−	−	−	0/5
PP-RP-10	634–642	AYYGRSVDF	100	+	−	−	+	−	2/5	−	−	−	−	−	−	0/5
CTL-inducible rate for each patient				6/10	2/10	6/10	5/10	6/10		0/10	0/10	0/10	0/10	0/10	0/10	

* A predicted half-time of peptide-dissociation from HLA class I molecules using BioInformatics and Molecular Analysis Section: HLA Binding Prediction, which is derived from Dr. Kenneth Parker's Research.[5]

† "+" indicates that the CTL line exhibited a significantly higher magnitude of cytotoxicity against PP-RP peptide-loaded C1R-A*2402 cells than against peptide-unloaded C1R-A*2402 cells ($P < 0.05$). The statistical significance was evaluated using Student's t test. These CTL lines were established from PBMCs stimulated with peptides for 4 weeks, and they also exhibited cytotoxic activity against tumor cells positive for both PP-RP and HLA-A24.

TE13shPP-RP cells (Fig. 7*B–E*). In the presence of relevant PP-RP–derived peptides, cytotoxic activity of the CTL lines against TE13shPP-RP cells markedly recovered. These findings clearly indicate that these CTL lines were specific to PP-RP and could lyse cancer cells in recognition of PP-RP–derived peptides in the context of HLA-A24 and that at least PP-RP-2, PP-RP-3, PP-RP-4, and PP-RP-8 peptides were naturally processed from PP-RP protein in the TE13 esophageal cancer cell line.

Inhibition of Esophageal Cancer Cell Growth in Nude Mice by Inoculation of a PP-RP–derived Peptide Induced CTL Line. The PP-RP-8–induced CTL line described above was expanded to a large number, and therapeutic efficacy was assessed in an experimental esophageal cancer xenograft model. After engrafting BALB/c nude mice s.c. with a human esophageal cancer cell line TE11, we did adoptive transfer experiments in which the PP-RP-8–induced human CTL line, control CD8$^+$ T-cell line, or PBS alone was inoculated into the tumor. Control CD8$^+$ T-cell line did not exhibit cytotoxicity against TE11 cells *in vitro* (data not shown). Growth curves of TE11 cells in mice are shown in Fig. 8*A*. Adoptive transfer of the PP-RP-8–induced CTL line resulted in a significant inhibition of tumor growth. The control T-cell line did not exhibit inhibitory effect on tumor growth *in vivo*, and PBS alone did not do so. Tumors in the mice inoculated with the PP-RP-8–induced CTL line were significantly smaller than those in control mice, and complete tumor regression was observed in one mouse given the CTL line. Survival curves of PP-RP-8–induced CTL-treated, control T-cell line-treated, or PBS-treated groups of mice are shown in Fig. 8*B*. A significant difference was observed among the three groups in that all of the mice in the control groups died within 120 days, whereas no mouse died in the PP-RP-8–induced CTL line-treated group within the same time period; hence, a marked prolongation of survival from cancer was observed in these mice ($P < 0.05$).

Reduced Proliferation of PP-RP–knockdown TE13 Cells. *PP-RP* gene maps on chromosome 16, at 16p12-p11.2 according to RefSeq. *PP-RP* mRNA is 5.9 kb long, and its premessenger covers 35.04 kb on the human genome. The protein sequence deduced from the genome sequence (1792 amino acid, M_r 201,000) contains an E3 ubiquitin ligase domain and a DNA topoisomerase I domain (Fig. 9*A*). PP-RP is predicted to localize in the nucleus.[4] To determine the intracellular localization of PP-RP, we carried out indirect immunofluorescence staining analysis using the PP-RP–positive cell line TE13. In mitotic cells, PP-RP colocalized with the chromosomes throughout mitotic stages (Fig. 9*B*). In interphase, strong PP-RP staining was detected throughout the nucleus, and weak staining was detected in the cytoplasm (Fig. 9*C*). No PP-RP staining was observed in SK-Hep1 cells, which did not express *PP-RP* mRNA.

We generated PP-RP stable knockdown TE13shPP-RP cells, as described above. After 2 months of culture, pSilencer-GFP–transfected control cells (TE13shGFP) were strongly stained with a PP-RP–specific antibody, whereas pSilencer-PP-RP–transfected cells showed a significant reduction in expression levels of PP-RP protein (Fig. 9*C*). PP-RP reduction was also confirmed in Western blot analysis, as described above (Fig. 7*A*). We then examined the proliferation rate when PP-RP was knocked down in TE13 cells. We investigated the growth of TE13 cells under low-serum culture conditions because cell proliferation has become independent of growth factors under such conditions. Fig. 9*D* shows the growth curve of a population of cells grown in 5% FCS. The effect was even more striking when cells were cultured in 1% FCS (Fig. 9*E*). The proliferation rate of TE13shPP-RP cells was significantly reduced as com-

[4] Internet address: http://psort.ims.u-tokyo.ac.jp/.

[5] Internet address: http://bimas.cit.nih.gov/molbio/hla_bind/.

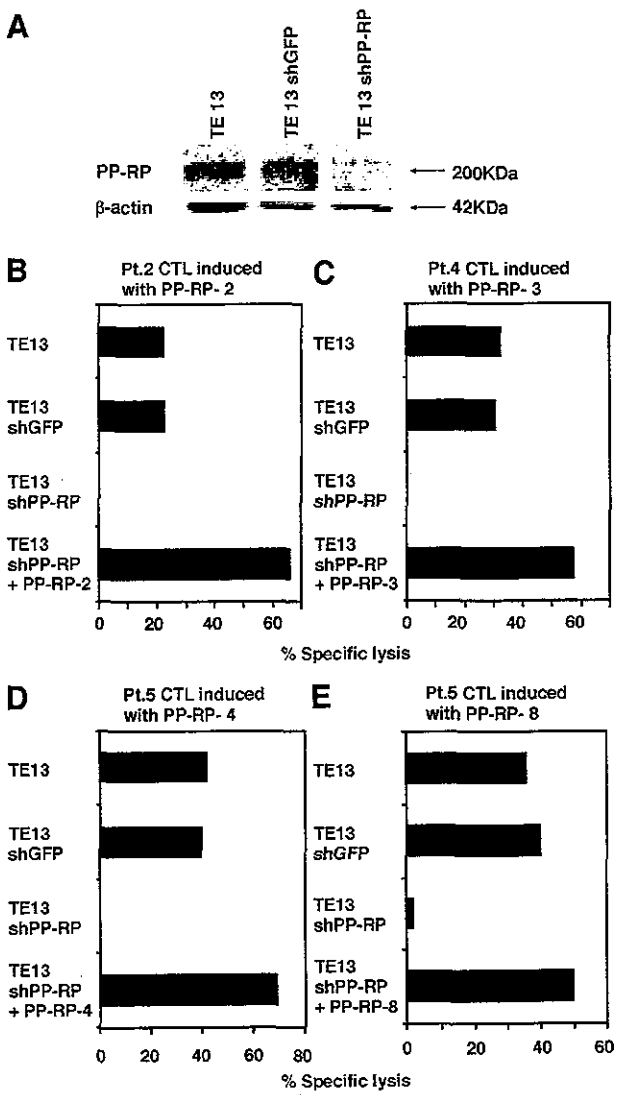

Fig. 7 Abrogation of PP-RP–specific CTL activity by down-regulation of PP-RP protein in TE13 target cells. *A*, down-regulation of PP-RP protein in TE13 cells transfected with pSilencer vector containing the human PP-RP siRNA TE13shPP-RP but not in control cells transfected with pSilencer vector containing GFP small interfering RNA, TE13shGFP. Western blotting analysis of whole cell lysates from TE13 cells (*Lane 1*), TE13shGFP cells (*Lane 2*), or TE13shPP-RP cells (*Lane 3*) using anti-PP-RP antibody confirmed the reduced expression of PP-RP protein in TE13shPP-RP cells. CTL lines used were the same as those shown in Figs. 5 and 6. Cytotoxic activities of CTL against TE13, TE13shGFP, TE13shPP-RP, or TE13shPP-RP prepulsed with relevant peptides were analyzed in a 4-hour ^{51}Cr release assay at *E:T ratio* of 20.

pared with wild-type and mock-transfected TE13 cells (~0.5-fold for PP-RP–knockdown cells). These data suggest that PP-RP–knockdown TE13 cells were more dependent on growth factors present in FCS and that PP-RP has important roles in promoting cell proliferation in TE13 esophageal cancer cells.

DISCUSSION

Identification of antigens naturally processed in and presented on tumor cells is important for the establishment of tumor immunotherapy. We identified *PP-RP* using cDNA microarray analysis of esophageal cancer. *PP-RP* was strongly expressed in esophageal cancer cells and in normal testis and placenta and weakly expressed in some normal tissues at the mRNA level. At the protein level, PP-RP was observed immunohistochemically in esophageal cancer cells and in normal testis and placenta but

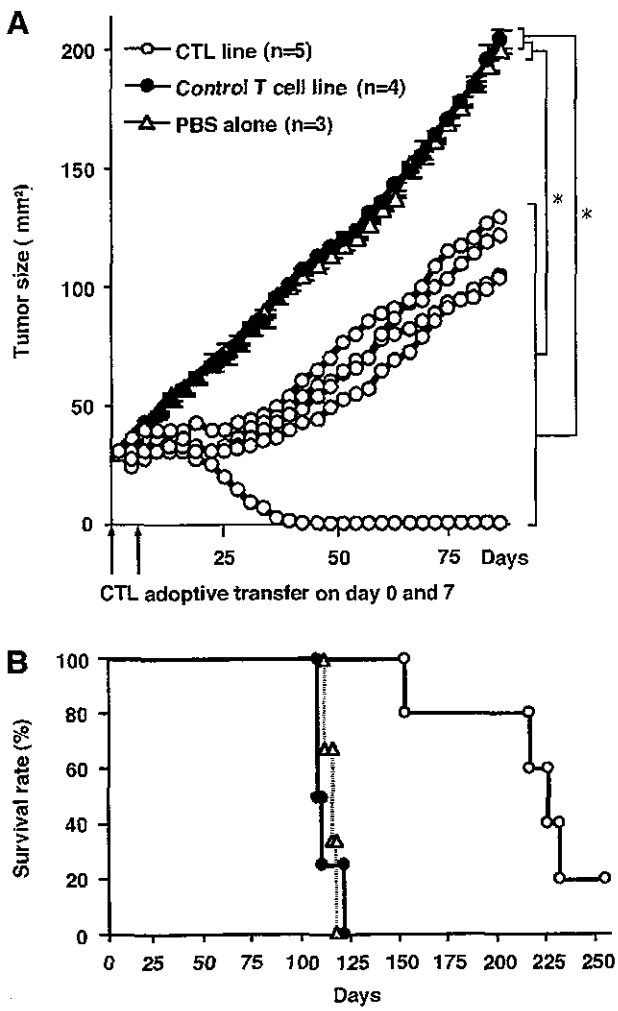

Fig. 8 *In vivo* antitumor activity of adoptively transferred PP-RP-8–induced CTLs. *A*, marked inhibition of growth of a human esophageal cancer cell line, TE11, engrafted into nude mice after adoptive CTL transfer. On day 0, tumor size was 30 mm^2, and CTLs (5×10^6) were inoculated into the tumor. On day 7, the same CTL inoculation was repeated. The control $CD8^+$ T-cell line, which did not show cytotoxicity against TE11, was also inoculated as a control. Tumor volumes in nude mice given two treatments on day 0 and day 7 with PP-RP-8–induced CTLs (○), control $CD8^+$ T-cell line (●), or PBS alone (△) are shown. Data are the mean ± SD for mice inoculated with control T-cell line (n = 4) or with PBS alone (n = 3). For five mice inoculated with the PP-RP-8–induced CTL line, tumor size of individual mouse is shown. Tumor size was expressed in square millimeters, and the statistical significance was evaluated using *t* test. *, $P < 0.05$. *B*, effect of adoptive CTL transfer on the survival of nude mice engrafted with TE11. The mice were given two treatments on day 0 and day 7 with PP-RP-8–induced CTLs (○), control $CD8^+$ T-cell line (●), or PBS alone (△ and *dashed line*). The prolonged survival of mice inoculated with PP-RP-8–induced CTLs was statistically significant ($P < 0.05$).

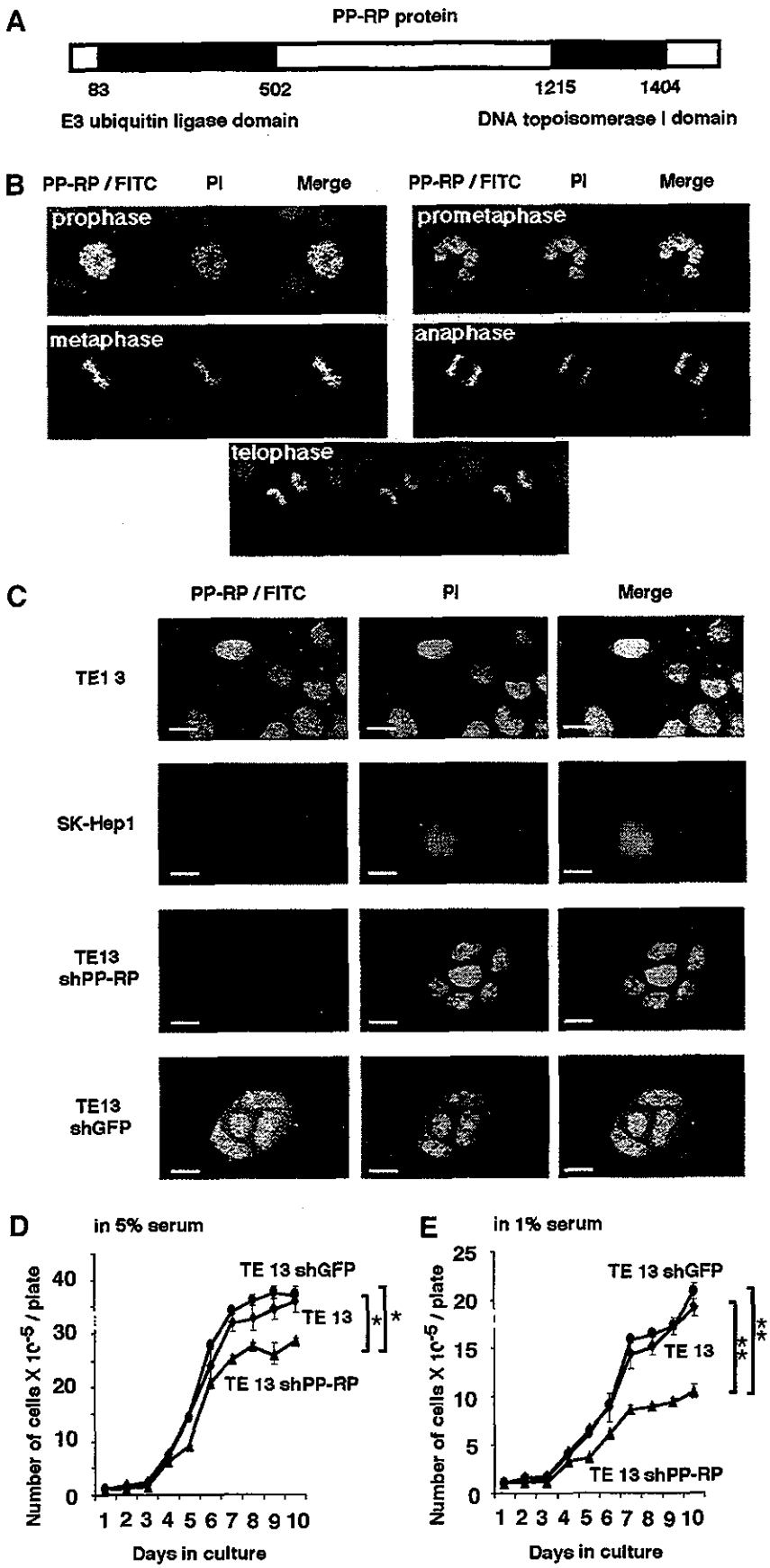

Fig. 9 Reduced proliferation of PP-RP–knockdown TE13 cells and colocalization of PP-RP with chromosomes. *A*, a scheme for domain composition of PP-RP protein. *B*, colocalization of PP-RP with chromosomes. TE13 cells in mitotic phases were fixed and stained with an anti-PP-RP antibody (PP-RP, *green*) and propidium iodide (PI, *red*), and analyzed using immunofluorescence microscopy. PP-RP was colocalized with chromosomes throughout the mitotic stages. *C*, reduced expression by RNA interference of the *PP-RP* gene in TE13 cells. Cells were analyzed, as described in *B*. SK-Hep 1 is shown as a PP-RP–negative cell, and TE13shGFP cells are also shown as a control. Down-regulation of PP-RP protein in TE13shPP-RP cells was confirmed. *Scale bars* represent 10 μm. *D* and *E*, reduced growth curve of TE13shPP-RP cells. Mock- and PP-RP–knockdown TE13 cells and normal TE13 cells were grown in RPMI 1640 supplemented with either 5% (*D*) or 1% (*E*) FCS, and cell numbers were counted daily. The data correspond to the mean values of three independent experiments ± SD, and the statistical significance was evaluated using *t* test. *, $P < 0.05$; **, $P < 0.01$.

not in other normal tissues. Because testis and placenta are immune privileged sites, PP-RP–specific CTLs can attack only esophageal cancer cells without injuring normal tissues in case of immunotherapy targeted on PP-RP. Thus, we chose PP-RP as a candidate of TAA for immunotherapy for patients with esophageal cancer.

We wanted to identify TAAs, which are involved in oncogenesis of esophageal cancer as a target for immunotherapy, because use of oncogenic TAAs may minimize the well-described risk of immune escape of cancer cells attributable to antigen loss, deletion, mutation, or down-regulation of TAAs as a consequence of therapeutically driven immune selection. We investigated oncogenic functions of PP-RP, which reportedly binds retinoblastoma protein (pRB; ref. 28). PP-RP contains multiple repeated sequences such as SRS, YRE, and VPPP. These regions involved in binding with pRB locate at a small region at the middle of PP-RP. Moreover, PP-RP contains both the E3 ubiquitin ligase domain and the DNA topoisomerase I domain and colocalizes with chromosomes in mitotic cells (Fig. 9). Both proliferation potential protein-related (P2P-R) and p53-associated cellular protein were reported to be mouse homologues of human PP-RP. P2P-R protein binds pRB (29) and colocalizes with chromosomes in mitotic cells (30).

The stable overexpression of P2P-R protein promotes camptothecin-induced apoptosis in the human breast cancer cell line, MCF-7 cells, and the specific region responsible for induction of apoptosis exists within amino acid residues 1156–1314 of P2P-R protein (31). p53-associated cellular protein is strongly expressed in normal testis and binds p53 and pRB (32). PP-RP consistently exhibits a low level of but a significant homology with MDM2. MDM2 is an E3 ubiquitin ligase that targets p53 to proteasomal degradation and localizes in the nucleus. The E3 ubiquitin ligase of MDM2 accelerates cell cycle progression (33, 34). The DNA mismatch repair system is involved in the correction of base/base mismatches and insertion/deletion loops arising during replication. DNA topoisomerase I has a critical role in the DNA mismatch repair system. It has been shown that some colorectal cancer cell lines exhibit an increased sensitivity to camptothecin, a human topoisomerase I inhibitor, which blocks DNA replication in human cancer cells (35), and camptothecin-induced apoptosis in gastric cancer cells (36). In the present study, we showed that cell growth rate was reduced in case of PP-RP–knockdown esophageal cancer cell line TE13 cells under low-serum culture condition (Fig. 9). Collectively, we are of the views that PP-RP has important roles in cell cycle progression in esophageal cancer cells, and additional investigations are planned to clarify oncogenic properties of PP-RP.

We found that in 15 cases of R1 group (patients with microscopic residual tumor after surgery) higher *PP-RP* expression was associated with a poor prognosis (Table 1 and Fig. 4). Thus, expression levels of *PP-RP* in esophageal cancer tissue may be a useful marker for the prediction of prognoses of the patients after surgery. Furthermore, the results suggest a possible involvement of PP-RP in the progression of esophageal cancer. Thus, immunotherapy targeting at PP-RP may be effective for such esophageal cancer patients with a poor prognosis.

In this study, we demonstrated that CTL lines specific to PP-RP–lysed tumor cells expressed PP-RP in a HLA-restricted manner (Figs. 5 and 6). These CTL lines could be generated *in vitro* from esophageal cancer patients but not from healthy donors, thus indicating that frequency of PP-RP–specific CTL precursors in esophageal cancer patients seemed to be higher than that in healthy donors. In general, tumors reject antigens such as NY-ESO-1, and melan-A/MART-1 contains two or three kinds of MHC-restricted CTL epitopes (37–41). On the other hand, PP-RP contains 10 peptides that can induce PP-RP–specific and HLA-A24–restricted CTL lines from five esophageal cancer patients (Table 2). The observation suggests that PP-RP can trigger an anticancer immune response even by breaking a previously established tolerance in esophageal cancer patients.

We identified PP-RP–derived peptides, which can be recognized by CTL in a HLA-A24–restricted manner. *HLA-A24* is the most common *HLA class I* allele in the Japanese population and ~60% of the Japanese, 33% of Chinese, 27% of Hispanics, and 17% of Caucasians are positive for *HLA-A24* (42). There are several methods for cell-mediated cancer immunotherapy, including peptide vaccination (43), immunization with dendritic cells pulsed with a peptide or tumor cell lysate (44, 45), immunization with dendritic cell/tumor cell hybrids (46), and adaptive transfer of tumor-specific CTL lines propagated *ex vivo* (47). In a previous study, WT1-specific CTLs were transferred i.v. into nude mice that had been engrafted with human lung cancer cells, and the efficacy of WT1-specific CTLs against human lung cancer was demonstrated (25). In the present study, we observed that transferred CTLs inhibited the growth of human esophageal cancer cells engrafted into nude mice. From this observation, we demonstrated that a locoregional administration treatment with PP-RP–reactive CTLs was effective in inhibiting growth of human esophageal cancer cells in nude mice. Endoscopic direct injection of PP-RP–reactive CTLs into tumors may prove to be a therapeutic intervention of esophageal cancer because intratumor injection of *in vitro* expanded CTLs has been demonstrated to be effective in clinical trials (48, 49). PP-RP may be an ideal TAA effective for cancer immunotherapy because PP-RP has immunogenicity and is expressed in cancer cells derived from >85% of esophageal cancer patients (Fig. 1*A*). The present findings suggest that adoptive transfer of PP-RP–specific CTLs may provide an effective treatment for esophageal cancer. Specifically, endoscopic intratumoral injection of these CTLs may be an effective method.

Finally, the demonstration of PP-RP–specific CTLs from cancer patients has important implications for ongoing efforts to characterize additional TAA. Unlike TAA defined by reactivity of T cells or immunoglobulin derived from cancer patients, the PP-RP epitopes described in this report were deduced from primary sequence and characterized by methods of reverse immunology (50). Although the method of antigen and epitope deduction has its own difficulties, advances in genomics and proteomics provide a growing number of candidate antigens strongly expressed in cancer cells among many cancer patients but rarely so in normal cells. As additional works in cancer biology characterize the function of these candidate genes, studies defining clinically relevant epitopes from these important candidate antigens can be guided by methods for characterization of PP-RP, as described in this study.

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REFERENCES

1. Tepper J. Refluxtions on esophageal cancer: can we swallow the changes? J Clin Oncol 2000;18:453–4.

2. Coia LR, Minsky BD, Berkey BA, et al. Outcome of patients receiving radiation for cancer of the esophagus: results of the 1992–1994 Patterns of Care Study. J Clin Oncol 2000;18:455–62.

3. Pouliquen X, Levard H, Hay JM, McGee K, Fingerhut A, Langlois-Zantin O. 5-Fluorouracil and cisplatin therapy after palliative surgical resection of squamous cell carcinoma of the esophagus. A multicenter randomized trial. French Associations for Surgical Research. Ann Surg 1996;223:127–33.

4. Schlag PM. Randomized trial of preoperative chemotherapy for squamous cell cancer of the esophagus. The Chirurgische Arbeitsgemeinschaft Fuer Onkologie der Deutschen Gesellschaft Fuer Chirurgie Study Group. Arch Surg 1992;127:1446–50.

5. Philip PA, Ajani JA. Has combined modality therapy improved the outlook in carcinoma of the esophagus? Oncology (Huntingt.) 1994;8: 37–42; discussion 44–5, 50–2, 61.

6. Nakazato H, Koike A, Saji S, Ogawa N, Sakamoto J. Efficacy of immunochemotherapy as adjuvant treatment after curative resection of gastric cancer. Study Group of Immunochemotherapy with PSK for Gastric Cancer. Lancet 1994;343:1122–6.

7. Ueda Y, Yamagishi H, Tanioka Y, et al. Clinical application of adoptive immunotherapy and IL-2 for the treatment of advanced digestive tract cancer. Hepatogastroenterology 1999;46 Suppl 1:1274–9.

8. Nakao M, Yamana H, Imai Y, et al. HLA A2601-restricted CTLs recognize a peptide antigen expressed on squamous cell carcinoma. Cancer Res 1995;55:4248–52.

9. Toh U, Yamana H, Nakao M, et al. HLA class I-restricted and tumor-specific cytotoxic T lymphocytes from metastatic lymph nodes of esophageal cancers. Cell Immunol 1997;177:137–43.

10. Azuma K, Shichijo S, Maeda Y, et al. Mutated p53 gene encodes a nonmutated epitope recognized by HLA-B*4601-restricted and tumor cell-reactive CTLs at tumor site. Cancer Res 2003;63:854–8.

11. Maeda Y, Ito M, Harashima N, et al. Cleavage and polyadenylation specificity factor (CPSF)-derived peptides can induce HLA-A2–restricted and tumor-specific CTLs in the majority of gastrointestinal cancer patients. Int J Cancer 2002;99:409–17.

12. Ito M, Shichijo S, Tsuda N, et al. Molecular basis of T-cell–mediated recognition of pancreatic cancer cells. Cancer Res 2001;61: 2038–46.

13. Nakatsura T, Senju S, Ito M, Nishimura Y, Itoh K. Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method. Eur J Immunol 2002;32:826–36.

14. Monji M, Senju S, Nakatsura T, et al. Head and neck cancer antigens recognized by the humoral immune system. Biochem Biophys Res Commun 2002;294:734–41.

15. Nakatsura T, Senju S, Yamada K, Jotsuka T, Ogawa M, Nishimura Y. Gene cloning of immunogenic antigens overexpressed in pancreatic cancer. Biochem Biophys Res Commun 2001;281:936–44.

16. Kihara C, Tsunoda T, Tanaka T, et al. Prediction of sensitivity of esophageal tumors to adjuvant chemotherapy by cDNA microarray analysis of gene-expression profiles. Cancer Res 2001;61:6474–9.

17. Karaki S, Kariyone A, Kato N, Kano K, Iwakura Y, Takiguchi M. HLA-B51 transgenic mice as recipients for production of polymorphic HLA-A, B-specific antibodies. Immunogenetics 1993;37:139–42.

18. Yang D, Nakao M, Shichijo S, et al. Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24–restricted cytotoxic T lymphocytes in cancer patients. Cancer Res 1999;59:4056–63.

19. Nakatsura T, Yoshitake Y, Senju S, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 2003;306:16–25.

20. Nakatsura T, Kageshita T, Ito S, et al. Identification of glypican-3 as a novel tumor marker for melanoma. Clin Cancer Res 2004;10: 6612–21.

21. Monji M, Nakatsura T, Senju S, Yoshitake Y, et al. Identification of a novel cancer/testis antigen, KM-HN-1, recognized by cellular and humoral immune responses. Clin Cancer Res. In press 2004.

22. Gomi S, Nakao M, Niiya F, et al. A cyclophilin B gene encodes antigenic epitopes recognized by HLA-A24–restricted and tumor-specific CTLs. J Immunol 1999;163:4994–5004.

23. Masuda M, Senju S, Fujii S, et al. Identification and immunocytochemical analysis of DCNP1, a dendritic cell-associated nuclear protein. Biochem Biophys Res Commun 2002;290:1022–9.

24. Matsuyoshi H, Senju S, Hirata S, Yoshitake Y, Uemura Y, Nishimura Y. Enhanced priming of antigen-specific CTLs in vivo by embryonic stem cell-derived dendritic cells expressing chemokine along with antigenic protein: application to antitumor vaccination. J Immunol 2004;172:776–86.

25. Makita M, Hiraki A, Azuma T, et al. Antilung cancer effect of WT1-specific cytotoxic T lymphocytes. Clin Cancer Res 2002;8:2626–31.

26. Katahira T, Nakamura H. Gene silencing in chick embryos with a vector-based small interfering RNA system. Dev Growth Differ 2003; 45:361–7.

27. Gupta S, Schoer RA, Egan JE, Hannon GJ, Mittal V. Inducible, reversible, and stable RNA interference in mammalian cells. Proc Natl Acad Sci USA 2004;101:1927–32.

28. Sakai Y, Saijo M, Coelho K, Kishino T, Niikawa N, Taya Y. cDNA sequence and chromosomal localization of a novel human protein, RBQ-1 (RBBP6), that binds to the retinoblastoma gene product. Genomics 1995;30:98–101.

29. Witte MM, Scott RE. The proliferation potential protein-related (P2P-R) gene with domains encoding heterogeneous nuclear ribonucleoprotein association and Rb1 binding shows repressed expression during terminal differentiation. Proc Natl Acad Sci USA 1997;94:1212–7.

30. Gao S, Witte MM, Scott RE. P2P-R protein localizes to the nucleolus of interphase cells and the periphery of chromosomes in mitotic cells which show maximum P2P-R immunoreactivity. J Cell Physiol 2002;191:145–54.

31. Gao S, Scott RE. Stable overexpression of specific segments of the P2P-R protein in human MCF-7 cells promotes camptothecin-induced apoptosis. J Cell Physiol 2003;197:445–52.

32. Simons A, Melamed-Bessudo C, Wolkowicz R, et al. PACT: cloning and characterization of a cellular p53 binding protein that interacts with Rb. Oncogene 1997;14:145–55.

33. Argentini M, Barboule N, Wasylyk B. The contribution of the RING finger domain of MDM2 to cell cycle progression. Oncogene 2000;19:3849–57.

34. Kawai H, Wiederschain D, Yuan ZM. Critical contribution of the MDM2 acidic domain to p53 ubiquitination. Mol Cell Biol 2003;23: 4939–47.

35. Jacob S, Aguado M, Fallik D, Praz F. The role of the DNA mismatch repair system in the cytotoxicity of the topoisomerase inhibitors camptothecin and etoposide to human colorectal cancer cells. Cancer Res 2001;61:6555–62.

36. Zhang ZW, Patchett SE, Farthing MJ. Topoisomerase I inhibitor (camptothecin)-induced apoptosis in human gastric cancer cells and the role of wild-type p53 in the enhancement of its cytotoxicity. Anticancer Drugs 2000;11:757–64.

37. Jager E, Chen YT, Drijfhout JW, et al. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2–binding peptide epitopes. J Exp Med 1998;187:265–70.

38. Coulie PG, Brichard V, Van Pel A, et al. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J Exp Med 1994;180:35–42.

39. Kawakami Y, Eliyahu S, Delgado CH, et al. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. Proc Natl Acad Sci USA 1994;91:3515–9.

40. Schneider J, Brichard V, Boon T, Meyer zum Buschenfelde KH, Wolfel T. Overlapping peptides of melanocyte differentiation antigen Melan-A/MART-1 recognized by autologous cytolytic T lymphocytes in association with HLA-B45.1 and HLA-A2.1. Int J Cancer 1998;75:451–8.

41. Castelli C, Storkus WJ, Maeurer MJ, et al. Mass spectrometric identification of a naturally processed melanoma peptide recognized by CD8+ cytotoxic T lymphocytes. J Exp Med 1995;181:363–8.

42. Date Y, Kimura A, Kato H, Sasazuki T. DNA typing of the HLA-A gene: population study and identification of four new alleles in Japanese. Tissue Antigens 1996;47:93–101.

43. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998;4:321–7.

44. Nestle FO, Alijagic S, Gilliet M, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 1998;4:328–32.

45. Geiger J, Hutchinson R, Hohenkirk L, McKenna E, Chang A, Mule J. Treatment of solid tumours in children with tumour-lysate–pulsed dendritic cells. Lancet 2000;356:1163–5.

46. Kugler A, Stuhler G, Walden P, et al. Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nat Med 2000;6:332–6.

47. Falkenburg JH, Wafelman AR, Joosten P, et al. Complete remission of accelerated phase chronic myeloid leukemia by treatment with leukemia-reactive cytotoxic T lymphocytes. Blood 1999;94: 1201–8.

48. Toh U, Yamana H, Sueyoshi S, et al. Locoregional cellular immunotherapy for patients with advanced esophageal cancer. Clin Cancer Res 2000;6:4663–73.

49. Toh U, Sudo T, Kido K, et al. Locoregional adoptive immunotherapy resulted in regression in distant metastases of a recurrent esophageal cancer. Int J Clin Oncol 2002;7:372–5.

50. Schultze JL, Vonderheide RH. From cancer genomics to cancer immunotherapy: toward second-generation tumor antigens. Trends Immunol 2001;22:516–23.

Featured Article

Identification of a Novel Human Cancer/Testis Antigen, KM-HN-1, Recognized by Cellular and Humoral Immune Responses

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ABSTRACT

Purpose: **We used serologic screening of a cDNA expression library of human testis to identify novel cancer/testis antigens that elicit both humoral and cellular immune responses in cancer patients.**

Experimental Design and Results: **We identified a novel gene designated *KM-HN-1* the expression of which is testis-specific among normal tissues; it contains coiled coil domains and a leucine zipper motif and encodes a putative protein consisting of 833 amino acids. KM-HN-1 expression was observed in various cancer tissues and cancer cell lines at both mRNA and protein levels. Immunofluorescence staining of an esophageal cancer cell line revealed that KM-HN-1 protein was present exclusively in the nucleus during mitosis. Recombinant KM-HN-1 protein was produced, and used for ELISA to quantitate levels of IgG antibody specific to KM-HN-1. Higher levels of IgG antibodies specific to KM-HN-1 were detected in many types and numbers of cancer patients but not in healthy donors. The CTL lines specific to KM-HN-1, generated from *HLA-A*2402*–positive healthy donors and cancer patients, killed human leukocyte antigen (HLA)-A24-positive cancer cells expressing KM-HN-1 but not cell lines that did not express either KM-HN-1 or HLA-A24.**

Conclusions: **We identified a novel cancer/testis antigen, KM-HN-1, which elicited humoral immune responses in patients with various types of cancer. Furthermore, KM-HN-1–specific CTLs could be generated from both healthy donors and cancer patients, which indicated that KM-HN-1 can be a candidate for an ideal target for cancer immunotherapy.**

INTRODUCTION

Identification of tumor antigens capable of inducing an anticancer immune response in cancer patients and development of immunogenic cancer vaccines targeting these antigens represent formidable tasks confronting tumor immunologists (1). In the early 1990s, van der Bruggen *et al.* (2) and Traversari *et al.* (3) reported the first successful cloning of a human tumor antigen, termed melanoma antigen-1 or MAGE-1 (subsequently renamed MAGE-A1), that elicited a spontaneous CTL response in an autologous melanoma patient. Subsequent analysis of normal tissues showed that MAGE-1 is expressed exclusively in normal testis. The tumor expression of MAGE-1, however, was not restricted to melanoma, rather it was also expressed in a remarkable proportion of various cancer cells (4). The testis is an immune privileged organ because spermatogenic cells do not express human leukocyte antigen (HLA) class I and II molecules at the cell surface (5). Concomitantly, the testis has a so-called blood–testis barrier in the seminiferous tubuli generated by Sertoli cells. The ectopic expression of cancer/testis (CT) antigens may thus lead to an autologous cellular and/or humoral immune response (6). Recognizing these striking features, L. J. Old (7) proposed the term CT antigens to encompass this heterogeneous group of antigens, and a CT nomenclature system was also proposed. CT antigens are thought to be an ideal target of cancer immunotherapy. Twenty CT antigen genes or gene families have been identified to date, but coordinated humoral and cellular immune responses have been reported for only a few CT antigens, including MAGE-A1, MAGE-A3, and NY-ESO-1 (6, 8).

The screening of tumor-derived expression libraries for antigens detected by high-titer IgG antibodies from sera of patients with cancer by serological analysis of recombinant tumor cDNA expression libraries with autologous serum (SEREX; serologic identification of antigens by recombinant expression cloning) allows for a systematic search of antigens of human cancers (9). In their initial application of this method, van der Bruggen *et al.* identified MAGE-A1 (2) and Brichard *et al.* identified tyrosinase (10), two antigens originally cloned as CTL targets, which indicated that SEREX can detect tumor antigens that elicit a CTL-mediated immune response (9). The successful cloning of SSX (11) and NY-ESO-1 (12) prompted a series of SEREX studies in various tumor types at many institutes. Because known CT antigens are expressed in only a small

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spectrum of human cancers and in only a fraction of cases of a given tumor type, identification of additional CT antigens is necessary.

The fact that all CT antigens are also expressed at high levels in testis prompted us to do serologic screening of a testis cDNA expression library enriched for specific transcripts instead of a cDNA library derived from tumor cells. This approach led to identification of several more CT antigens (13). Herein, we report the expression pattern of, and humoral and cellular immune response to, KM-HN-1, a novel CT antigen that we identified with this approach.

MATERIALS AND METHODS

Patients, Tumor Tissues, and Cell Lines. Sera and tumor tissues were obtained during routine diagnostic procedures after obtaining a formal agreement signed by the patients. COS-7 and HSC-4 cells were used for the transfection. The human B lymphoblastoid cell line C1R, expressing a trace amount of HLA class I molecule and C1R-*A*2402* [a *HLA-A*2402* transfectant of C1R cells (14)] were used for peptide-pulse experiments. The other cell lines used in this study were as follows: lung carcinoma (PC-9), esophageal carcinomas (*TE3, TE9, TE11,* and *TE13*), and head and neck carcinomas (HSC-4 and HSQ-89). The origins and *HLA* genotypes of these cell lines have been described elsewhere (15–17).

Cloning and Production of Recombinant Protein of KM-HN-1. Immunologic screening was done as described previously (18). Briefly, cDNA libraries from cell lines of squamous cell carcinoma of head and neck (SCCHN) and a normal testicle tissue were screened with sera from six allogeneic SCCHN patients. A total of 25 positive clones belonging to 19 different genes, including *MAGE-A4* and *NY-LU-5*, were identified, and details of these genes were described elsewhere (18). Among these clones, a novel gene, *KM-HN-1*, was expressed only in testis among normal tissues but was expressed in several cancer tissues. So we further investigated KM-HN-1, its features and immunogenicity.

Reverse transcription-PCR (RT-PCR) analysis of normal and cancer tissue was done as described previously (18). Briefly, 1 μg of total RNA was isolated from each sample and converted into cDNA in 20 μL of reaction buffer. PCR was done for 30 cycles for quantification of *KM-HN-1* mRNA and for 25 cycles for quantification of β-*actin* mRNA. KM-HN-1 PCR primer sequences were: sense 5′-CCATCCCAGATACATTCCGAGGAC-3′ and antisense 5′-GGTCGAGGAAGGACAGTGTGT-3′. RT-PCR was done in at least two independent experiments. Quantitative RT-PCR was carried out in a Roche Lightcycler (Roche Diagnostics, Lewes, United Kingdom) and was quantified by measuring SYBR green. PCR was done with the DNA Master SYBR green I PCR reaction mix that contains *Taq* polymerase, dNTP mix (with dUTP instead of dTTP; *Roche*), 3 *mmol/L* $MgCl_2$ with a 0.5 μmol/L concentration of each primer, and either 1 μL of cDNA or 1 μL of water in a total volume of 20 μL. The PCR cycles were 95°C for 10 minutes, followed by 45 cycles of 95°C for 1 second, 68°C for 1 second, and 72°C for 10 seconds. KM-HN-1 PCR primer sequences were as follows: sense 5′-CCATCCCAGATACATTCCGAGGAC-3′ and antisense 5′-CTGATTTCCGACTGTACCATGCTG-3′. Quantification was achieved by comparison with an internal standard curve containing 10-fold dilutions of testis cDNA probe. The relative expression of the *KM-HN-1* mRNA was calculated with the following formula (19):

$$\text{Ratio} = \frac{(E_{\text{KM-HN-1}})^{\Delta CP\text{KM-HN-1}(\text{testis-sample})}}{(E_{\beta\text{-actin}})^{\Delta CP\beta\text{-actin}(\text{testis-sample})}} \times 100$$

Northern blot analysis was done also, as described previously (20). Integrity of RNA was checked by electrophoresis in formalin-MOPS (4-morpholinepropanesulfonic acid) gels. Gels with 20 μg of total RNA per lane were blotted onto nylon membranes. *KM-HN-1*–specific cDNA probe (bp 231–789) was used.

To prepare KM-HN-1–glutathione*S*- transferase (KM-HN-1GST) fusion protein, a1466-bp DNA fragment that was digested from a Homo sapiens cDNA clone IMAGE 4825416 (GenBank accession no. BG724227) and that corresponded to nucleotide position 149-1614 of *KM-HN-1c* DNA, was inserted into a prokaryotic expression vector pGEX-4T-3 (Amersham Pharmacia Biotech, Piscataway, NJ). This KM-HN-1 fragment covers NH_2-terminal 488 amino acids (58.6%) of the whole KM-HN-1 consisting of 833 amino acids (Fig.1). Fusion protein synthesis was done as described previously(21) and was used for ELISA and for preparation of an anti-KM-HN-1 antibody by immunizing rabbits with KM-HN-1 GST fusion protein. The antiserum was affinity purified, with KM-HN-1 GST fusion protein and GST protein chromatography columns.

Immunocytochemical and Immunohistochemical Analyses. In immunocytochemical analysis, we used COS-7 and TE13 cell lines. To construct a mammalian expression vector,

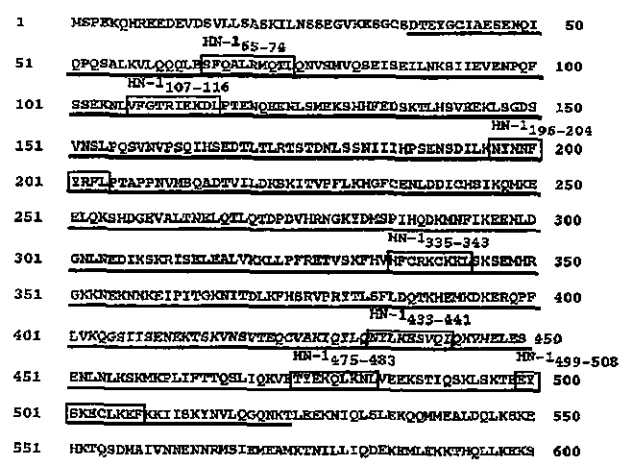

Fig. 1 Predicted amino acid sequences of human KM-HN-1 protein. *Underlined amino acid sequence* corresponds to a recombinant KM-HN-1 protein produced as GST fusion protein; *open box*, synthetic peptides, which have binding motifs for *HLA-A*2402*–encoded molecules, for use of CTL generation.

we inserted full-length *KM-HN-1* cDNA into pCAGGS-IRES-neo-R, downstream of the CAG promoter (22, 23). COS-7 cells were transfected with the construct by lipofection with LipofectAMINE 2000 Reagent (Invitrogen Corp., Carlsbad, CA). Transfection and staining were done as described previously (24). We used FITC-labeled goat antirabbit IgG as a second antibody and propidium iodide for nuclear DNA staining. A confocal microscope (Fluoview FV300, Olympus) was used for observation. Immunohistochemical examinations were done as described previously (25). We stained 4-μm-thick sections of formalin-fixed and paraffin-embedded tissue samples with anti-KM-HN-1 antibody at a dilution of 1:20.

Detection by ELISA of a KM-HN-1–specific Antibody in Serum Samples. Detection and titration of an antibody specific to a NH_2-terminal half fragment of KM-HN-1 was done, with indirect ELISA as described previously (21). Briefly, microtiter plates (96-well; NUNC, Roskilde, Denmark) were coated with GST-KM-HN-1 fusion protein in PBS (pH 7.4) for 16 hours at 4°C. The plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and were blocked with 5% skim milk/PBS for 1 hour at room temperature. The plates were then washed and incubated for 2 hours at room temperature with serum samples diluted at 1:100 with 1% skim milk/PBS. The plates were washed, and 100 μL of horseradish peroxidase-conjugated mouse antihuman IgG diluted at 1:2000 was added to each well, followed by incubation at room temperature for 1 hour. The plates were washed, and then 100 μL of solution of *o*-phenylenediamine (Sigma Fast, Sigma Chemical Co., St. Louis, MO) were added to each well. After 30 minutes, the reaction was stopped by adding 100 μL of 3 mol/L H_2SO_4, and absorbance (*A*) at 490 nm was determined, with a Model 550 microplate reader (Bio-Rad, Hercules, CA). In addition to GST-KM-HN-1 fusion proteins as coating antigens, each serum sample was tested for reactivity against GST alone, as a control for nonspecific binding. The ratio of $A_{GST\text{-}KM\text{-}HN\text{-}1}$ to A_{GST} was calculated to express the degree of KM-HN-1–specific reactivity of antibodies above background. An absorbance ratio exceeding two SDs above the mean ratio of $A_{GST\text{-}KM\text{-}HN\text{-}1}$ to A_{GST} in 16 normal donors sera (2.15) was interpreted to be positive. Specificity of each positive sample was examined by testing reactivity after preincubation with GST-KM-HN-1. Confirmed positive samples had decreased GST-KM-HN-1 reactivity on ELISA compared with nonpreincubated sera. Distributions of absorbance values between healthy donors and various types of cancer patients were compared with two-tailed Student's *t* test. The statistical significance of the difference in ratio of seropositive donors between cancer patients and healthy donors was assessed with a χ^2 test.

Generation of KM-HN-1 Peptide-Specific CTL Lines. We searched for synthetic peptides (purity, >90%), derived from the sequence of KM-HN-1 with binding motifs for *HLA-A*2402*–encoded molecules (including tyrosine or phenylalanine at position 2 and isoleucine, leucine, or phenylalanine at position 9 or 10), with BIMAS software (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD) and obtained from Bio-Synthesis, Inc. (Lewisville, TX; Fig. 1). An HIV nef-derived peptide (RYPLTFGWCF) that can bind to *HLA-A*2402*–encoded molecules was used as a negative control (26). Dendritic cells were generated as described previously (27). The generated cells expressed dendritic cell-associated molecules, such as CD1a, CD83, CD86, and HLA-DR, on their cell surfaces (data not shown). $CD8^+$ T cells were isolated, with CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), from peripheral blood mononuclear cells (PBMCs) of the same donors. A total of 2×10^6 $CD8^+$ T cells were cultured with 2×10^5 irradiated (3500 cGy) autologous dendritic cells in RPMI 1640, supplemented with 10% heat-inactivated autologous serum, 5 ng/mL recombinant human interleukin (IL)-7 (PeproTech EC Ltd, London, United Kingdom), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine, together with a KM-HN-1 synthetic peptide at a concentration of 1 μg/mL in a 16-mm-well plate. After culturing for 7 days, one half of the medium was exchanged for fresh culture medium supplemented with IL-7 and 10 units/mL recombinant human IL-2, and the cells were stimulated again by adding 2×10^5 irradiated (3500 cGy) autologous dendritic cells and KM-HN-1 peptide at a concentration of 1 μg/mL.

After a culture for an additional 7 days, the cells were stimulated a third time. The cells on day 20 of culture were tested for their peptide specificity by quantitating IFN-γ produced by CTLs, as described previously (28). Briefly, C1R-A*2402 cells (1×10^4), suspended in 100 μL RPMI 1640 supplemented with 10% fetal calf serum (assay medium), were seeded into round-bottomed microtiter wells and were incubated with or without synthetic peptide for 2 hours. Effector cells (1×10^5) suspended in 100 μL of assay medium were added to the well and were incubated for 16 hours; and the concentration of IFN-γ in the culture supernatants was measured with ELISA in duplicate assays. The KM-HN-1–specific CTL lines were cultured continuously in IL-2–containing culture medium, and irradiated autologous PBMCs and the KM-HN-1 peptide were added to the wells every week.

CTL Responses against Cancer Cell Lines. CTL response against cancer cell lines was measured by ELISA quantification of IFN-γ production as described above and by chromium release assay. Chromium release assay and cold target inhibition assay were done as described previously (29). Briefly, ^{51}Cr ($Na_2{}^{51}CrO_4$) labeled target cells (1×10^4) suspended in 100 μL of assay medium were seeded into round-bottomed 96-well plates. Various numbers of effector cells suspended in 100 μL of assay medium were added to the well and were incubated for 4 hours, and 100 μL of supernatant were collected from each well. In the cold target inhibition assay, various numbers of unlabeled- and peptide pulsed-C1R-A*2402 cells were added to the well. The percentage specific lysis was calculated as follows:

$$\frac{cpm\ experimental\ release - cpm\ spontaneous\ release}{cpm\ maximal\ release - cpm\ spontaneous\ release} \times 100$$

where cpm = count per minute. To investigate CTL response to IFN-γ-treated target cells, target cells were incubated with culture medium supplemented with 100 units/mL recombinant human IFN-γ for 48 hours before the assay.

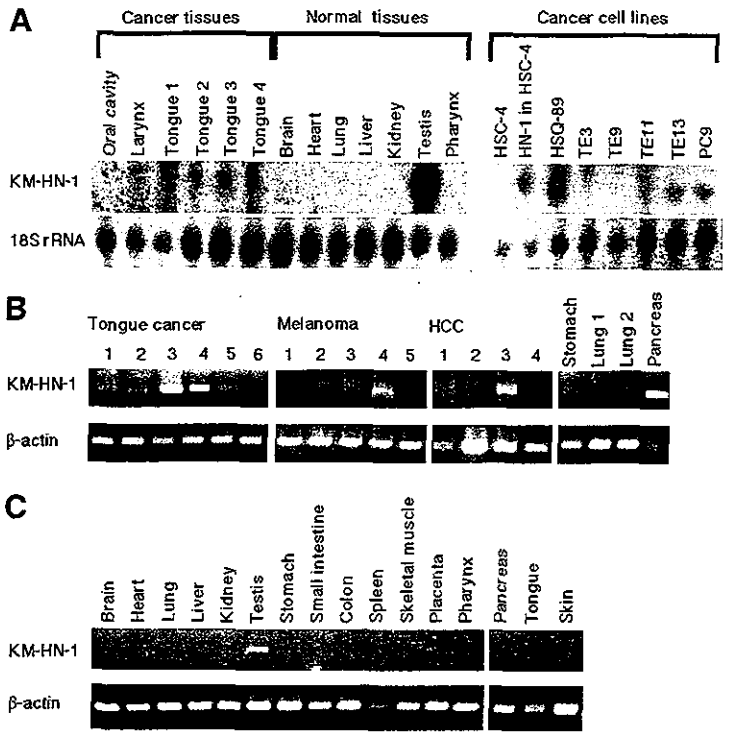

Fig. 2 Expression of *KM-HN-1* mRNA in normal and cancer tissues, and cancer cell lines. *A*, Northern blot analysis of *KM-HN-1* mRNA in various normal and cancer tissues, and cancer cell lines. The same filters were stripped and rehybridized with 18S rRNA to prove RNA integrity and to assess loading of equal amounts of RNA. RT-PCR analysis of *KM-HN-1* expression in various cancer tissues (*B*) and normal tissues (*C*). The same cDNA samples were tested for β-*actin* as an internal control.

RESULTS

Cloning of *KM-HN-1* cDNA. We identified a 2.9-kb cDNA clone designated as *KM-HN-1* (DNA Data Bank of Japan accession no. AB080722) after antibody-based screening of a cDNA expression library derived from normal testis with the use of serum from a patient with squamous cell carcinoma of the head and neck (SCCHN). This clone contained a 2499-bp complete open reading frame. The DNA sequence at the start codon in the open reading frame contained a Kozak consensus sequence (*A/GNNATGG*) for high efficiency protein translation (30). A polyadenylation signal (AATAAA) was found in the 3′untranslated region, which suggested that this transcript had a complete 3′untranslated region sequence upstream of the polyadenylate tail. Comparisons with the nucleotide sequence of a gene encoding for hypothetical protein MGC33607 revealed the *KM-HN-1* clone to be identical to MGC33607. This gene maps on chromosome 4, at 4q35.1 according to RefSeq.[5] KM-HN-1 contains a leucine zipper pattern, 3 coiled coil domains, and an endoplasmic reticulum membrane domain as judged by Psort 2 (http://psort.ims.u-tokyo.ac.jp/; Fig. 1).

KM-HN-1 Is Broadly Expressed in Cancer Tissues and Cancer Cell Lines but Expression in Normal Tissues Is Limited in Testis. As shown in Fig. 2*A*, a 2.9-kb transcript consistent with *KM-HN-1* was expressed only in testis among normal tissues and was expressed in four of six SCCHN tissues and in several cancer cell lines, in Northern blot analysis. In tumor tissues, *KM-HN-1* was expressed in a subset of a broad spectrum of tumors of different origins. As shown in Fig. 2*B*, some cancer tissues expressed a detectable amount of *KM-HN-1* mRNA. Analysis of expression levels of mRNA in 17 normal tissues by RT-PCR detected specific mRNA only in testis (Fig. 2*C*). *KM-HN-1* expression levels in various normal and cancer tissues were determined by quantitative RT-PCR, and these data were evaluated by calculating relative ratio to the *KM-HN-1* expression level in the human testis. Although relative *KM-HN-1* expression levels in all normal tissues except testis are under 0.02, those in five of six tongue cancers, three of five melanomas, one of four hepatocellular carcinomas (HCCs), one gastric cancer, and one pancreatic cancer tissues are above 0.1 (Table 1). Furthermore, expression of *KM-HN-1* mRNA was detected in various types of cancer cell lines established from SCCHN, esophageal cancer, lung cancer, gastric cancer, HCC, melanoma, pancreatic cancer, and colon cancer by RT-PCR analysis (Table 2).

Nuclear Localization of KM-HN-1 Protein. To determine the specificity of the anti-KM-HN-1 antibody, we carried out indirect immunofluorescence staining analysis with *KM-HN-1*–transfected COS-7 cells. COS-7 cells were transiently transfected with a *KM-HN-1* expression vector and were grown on fibronectin-coated coverslips. The cells were doubly stained with propidium iodide and anti-KM-HN-1 polyclonal antibody, and signals were detected with a FITC-labeled goat antirabbit IgG antibody. No KM-HN-1 staining signal was observed when COS-7 cells were transfected with a vector only. On the other hand, when *KM-HN-1* expression vector was used for transfection, about 20% of the COS-7 cells were stained (data not shown). Forced expression of *KM-HN-1* did not induce morphologic changes in COS-7 cells. To determine the subcellular

[5] Internet address: http://www.ncbi.nlm.nih.gov/RefSeq/.

Table 1 Relative expression levels of *KM-HN-1* mRNA in normal and cancer tissues

Cancer tissues	Relative ratio*	Normal tissues	Relative ratio
Tongue cancer			
1	0.5	Brain	ND
2	1.0	Heart	ND
3	5.0	Lung	ND
4	1.0	Liver	0.02
5	0.7	Kidney	ND
6	ND	Testis	100
Melanoma			
1	ND	Stomach	0.001
2	0.1	Small intestine	ND
3	0.5	Colon	ND
4	0.7	Spleen	0.004
5	ND	Skeletal muscle	ND
HCC			
1	ND	Placenta	ND
2	ND	Pharynx	0.002
3	4.0	Pancreas	0.01
4	ND	Tongue	ND
Gastric cancer	0.1	Skin	0.002
Lung cancer			
1	ND	PBMC	ND
2	ND	PHA blast	ND
Pancreatic cancer	2.0		

Abbreviations: ND, not determined; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin.

* The relative expression of the *KM-HN-1* mRNA was calculated using the following formula: ratio = $(E\text{KM-HN-1})^{\Delta CP_{\text{KM-HN-1}}(\text{testis-sample})}$ / $(E\beta\text{-actin})^{\Delta CP_{\beta\text{-actin}}(\text{testis-sample})} \times 100$.

localization of KM-HN-1 protein, we also stained TE13 cells (esophageal cancer cell lines expressing abundant intrinsic KM-HN-1 protein) with anti-KM-HN-1 antibody. In interphase, KM-HN-1 staining was detected throughout the nucleus, especially bright at the nulcear boundary with weak staining in the cytoplasm (Fig. 3*A–C*). During mitosis, KM-HN-1 colocalized with chromosomes (Fig. 3D–O).

Expression of KM-HN-1 Protein in Testis and Several Cancer Tissues. To determine whether the anti-KM-HN-1 antibody could also recognize KM-HN-1 expressed in paraffin-embedded tissues, we analyzed testicular tissue immunohistochemically. KM-HN-1 expression in mature testis was found to occur only in spermatogenic cells, mainly in the cytoplasm of spermatocytes and occasionally in that of spermatogonia (Fig. 4*A*). The expression was heterogeneous, and only a fraction of spermatocytes was stained at the nucleus. Neither interstitial cells nor Sertoli cells were stained with the anti-KM-HN-1 antibody. Using this antibody, we next examined expression of KM-HN-1 in several tumor tissues. KM-HN-1 was stained in 4 of 10 esophagus, 1 of 2 breast, and 1 of 3 colon cancer tissues (Fig. 4*E*, *F*, and *G*). In these cells, staining was observed mostly in the cytoplasm with nuclear staining being less prominent. In contrast, KM-HN-1 was not stained in the normal esophagus, breast, or colon tissues (Fig. 4*B*, *C*, and *D*).

Quantitation of Serum IgG Specific to KM-HN-1 in Healthy Donors and Cancer Patients. Because KM-HN-1 was widely expressed in a variety of tumor tissues and cell lines, we used ELISA to detect and quantitate levels of IgG antibody specific to KM-HN-1 in sera obtained from patients with a variety of solid tumors. As summarized in Fig. 5 and Table 2, the antibody was not detected in 16 healthy donors, but some patients with various types of cancer were positive for the antibody. Statistical significance of sero-positive ratio between cancer patients and healthy donors was observed except for melanoma and pancreatic cancer. A statistical significance of difference in distributions of anti-KM-HN-1 IgG levels between cancer patients and healthy donors was observed except for HCC and pancreatic cancer (Table 2). In each instance in which reactivity against GST-KM-HN-1 was greater than reactivity

Table 2 Summary for expression and immunogenicity of KM-HN-1

	KM-HN-1 expression (RT-PCR)* No. positive/no. tested		Anti-KM-HN-1 antibody†					
				Absorbance ratio†		No. positive/	*P* value	
Tissue type	Tumor tissues	Cell lines	Age mean	Mean	SD	no. tested‡	χ^2 test‡	t-tes t§
Healthy donor	—	—	63	1.49	0.33	0/16	—	—
SCCHN	4/6	5/12	59	2.05	0.49	6/21	<0.05	<0.01
Esophageal cancer	n.t.	8/9	59	4.02	2.28	6/6	<0.01	<0.01
Lung cancer (adeno)	1/2	2/4	64	2.26	0.72	4/7	<0.01	<0.01
Breast cancer	n.t.	n.t.	50	2.11	0.51	5/10	<0.01	<0.01
Gastric cancer	1/1	2/2	71	2.55	1.07	6/14	<0.01	<0.01
HCC	2/4	5/7	68	3.27	4.94	4/14	<0.05	>0.05
Biliary cancer	n.t.	n.t.	70	2.05	0.55	2/6	<0.05	<0.01
Pancreatic cancer	1/1	2/2	58	1.81	0.52	2/11	>0.05	>0.05
Colon cancer	n.t.	2/2	66	2.29	0.58	12/21	<0.01	<0.01
Melanoma	3/5	4/7	59	1.93	0.26	4/28	>0.05	<0.01

Abbreviation: n.t., not tested.

* Expression levels of mRNA were analyzed by RT-PCR with oligonucleotides specific for *KM-HN-1*.

† Antibody titer was determined with indirect ELISA with KM-HN-1-GST fusion protein.

‡ The statistical significance of difference in ratios of sero-positive donors between cancer patients and healthy donors was assessed using a χ^2 test. The cutoff value (2.15) is the mean plus two SD for healthy donors' sera.

§ The statistical significance of difference in distributions of Absorbance values between healthy donors and various types of cancer patients was compared by two-tailed Student's *t* test.