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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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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

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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

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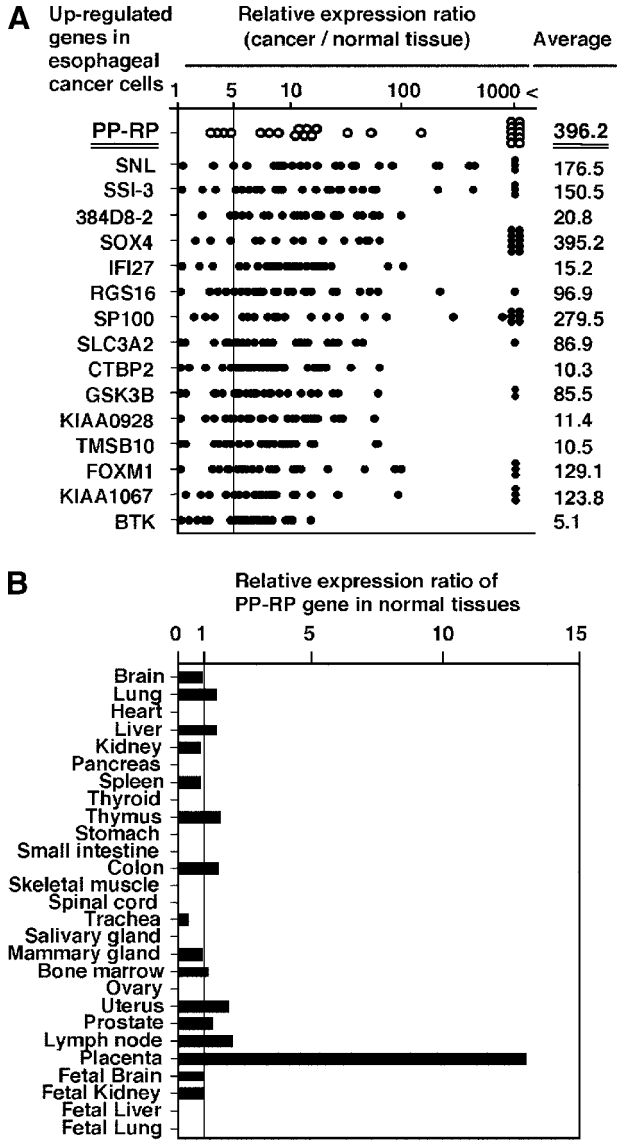

Fig. 1 Markedly enhanced expression of *PP-RP* mRNA in esophageal cancer tissues as based on cDNA microarray analysis. *A*, a list of up-regulated genes in esophageal cancer cells (16). These genes were expressed more than five times higher in cancer cells as compared with normal counterparts in >20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

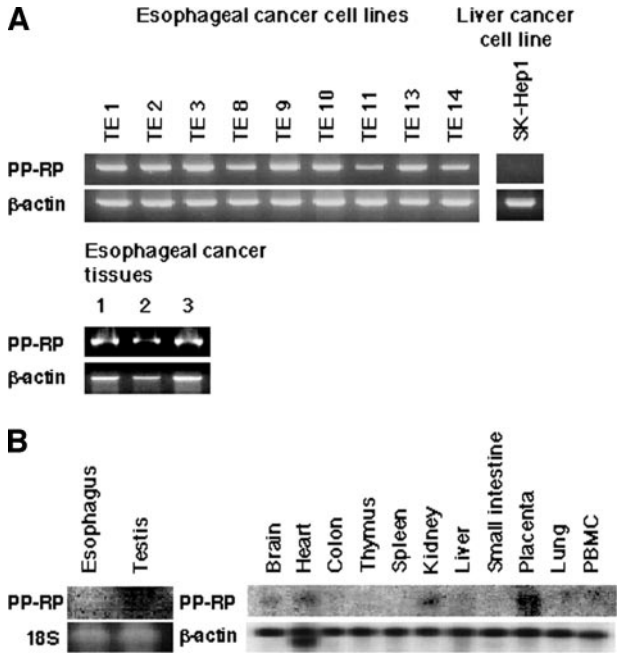

Fig. 2 Expression of *PP-RP* mRNA in cancer cell lines, cancer tissues, and normal tissues. *A*, RT-PCR analysis of *PP-RP* expression in various cancer cell lines and three esophageal cancer tissues. The same cDNA samples were tested for β-*actin* expression as a control. RT-PCR was done in at least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

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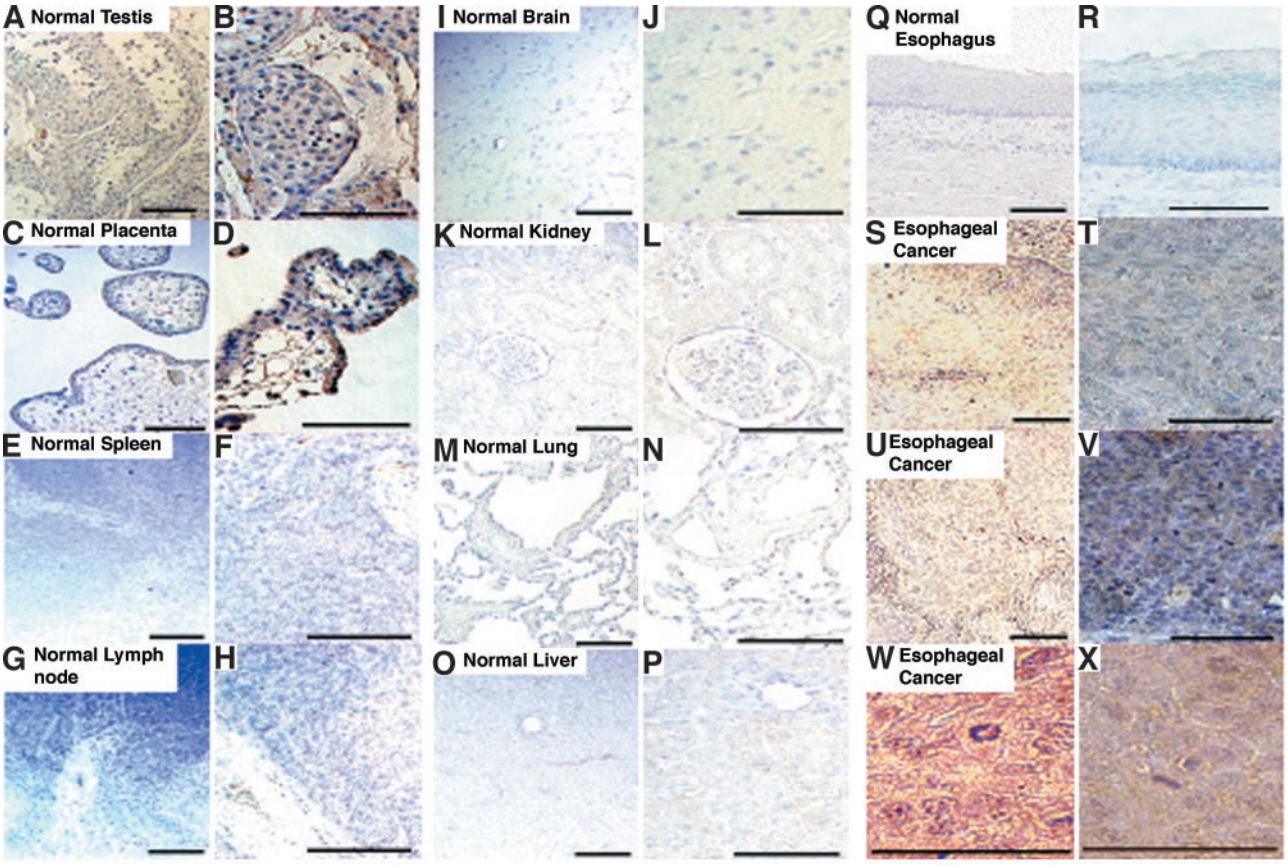

Fig. 3 Immunohistochemical staining of PP-RP protein. Normal testis (*A* and *B*), normal placenta (*C* and *D*), normal spleen (*E* and *F*), normal lymph node (*G* and *H*), normal brain (*I* and *J*), normal kidney (*K* and *L*), normal lung (*M* and *N*), normal liver (*O* and *P*), normal esophagus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

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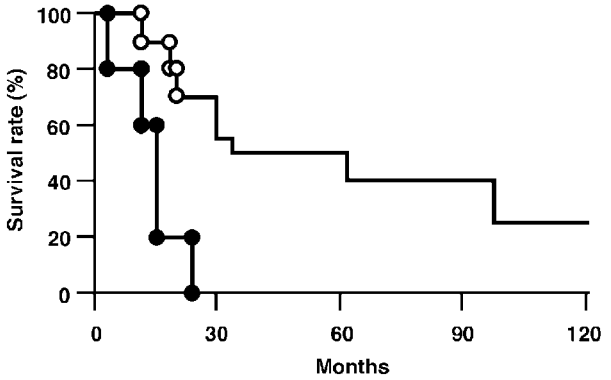

Fig. 4 Association between higher expression levels of the *PP-RP* gene and poor prognoses after surgery in 15 R1 esophageal cancer patients. All patients of R1A group, with very high *PP-RP* expression in cancer cells, died within 24 months (●). In contrast, 7 of 10 patients of R1B group (relative *PP-RP*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

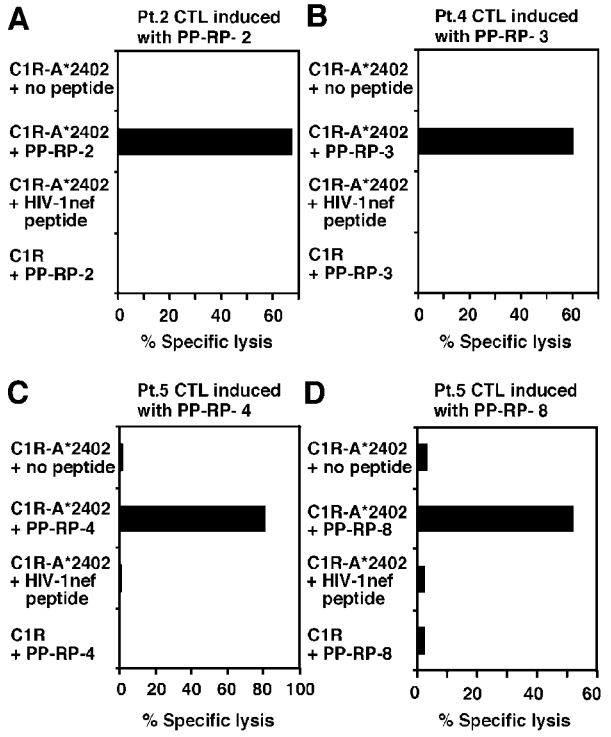

Fig. 5 PP-RP–derived peptide-specific and HLA-A*2402–restricted cytotoxicity of CTL lines. CTL lines specific to PP-RP-2 (*A*), PP-RP-3 (*B*), PP-RP-4 (*C*), or PP-RP-8 (*D*) were generated from esophageal cancer patients 2, 4, 5, or 5, respectively. Cytotoxic activities of CTL lines against C1R or *A*2402*-transfected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

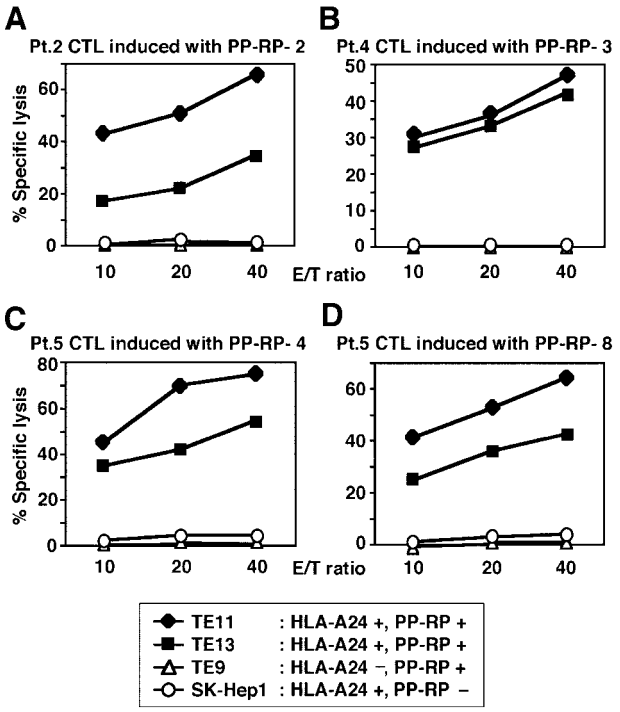

Fig. 6 Cytotoxic activities of peptide-induced CTL lines against cancer cell lines. CTL lines used were the same as those shown in Fig. 5. Cytotoxic activities of CTL lines against cancer cell lines were detected in a 4-hour ^{51}Cr release assay at the indicated E:T ratios. An assay was set up in duplicate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

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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/.

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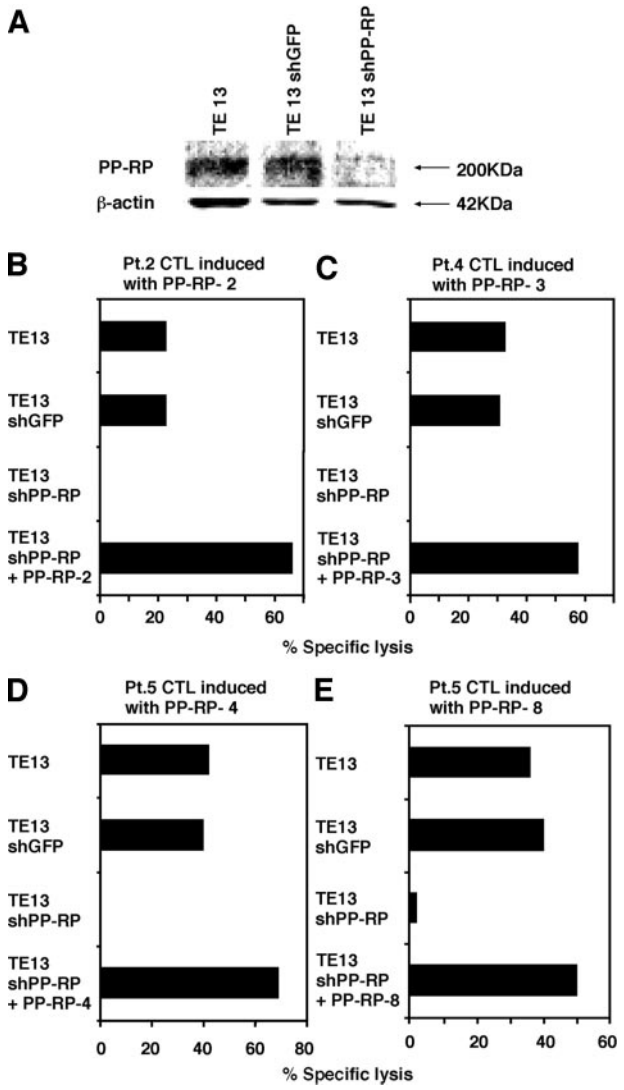

Fig. 7 Abrogation of PP-RP–specific CTL activity by down-regulation of PP-RP protein in TE13 target cells. *A*, down-regulation of PP-RP protein in TE13 cells transfected with pSilencer vector containing the human PP-RP siRNA TE13shPP-RP but not in control cells transfected with pSilencer vector containing 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

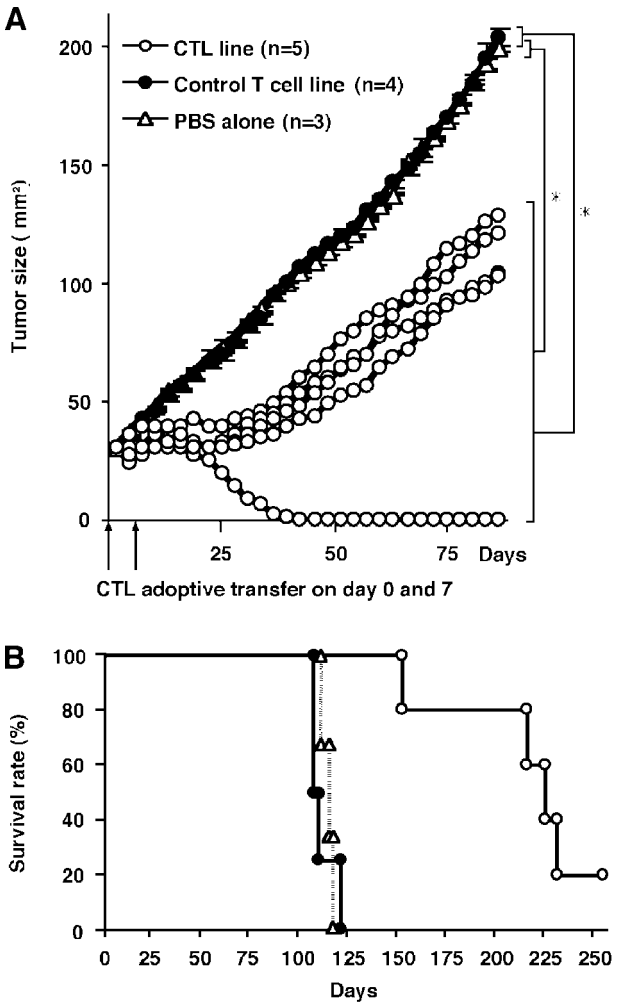

Fig. 8 *In vivo* antitumor activity of adoptively transferred PP-RP-8–induced CTLs. *A*, marked inhibition of growth of a human esophageal cancer cell line, TE11, engrafted into nude mice after adoptive CTL transfer. On day 0, tumor size was 30 mm^2, and CTLs (5×10^6) were inoculated into the tumor. 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 ($P < 0.05$).

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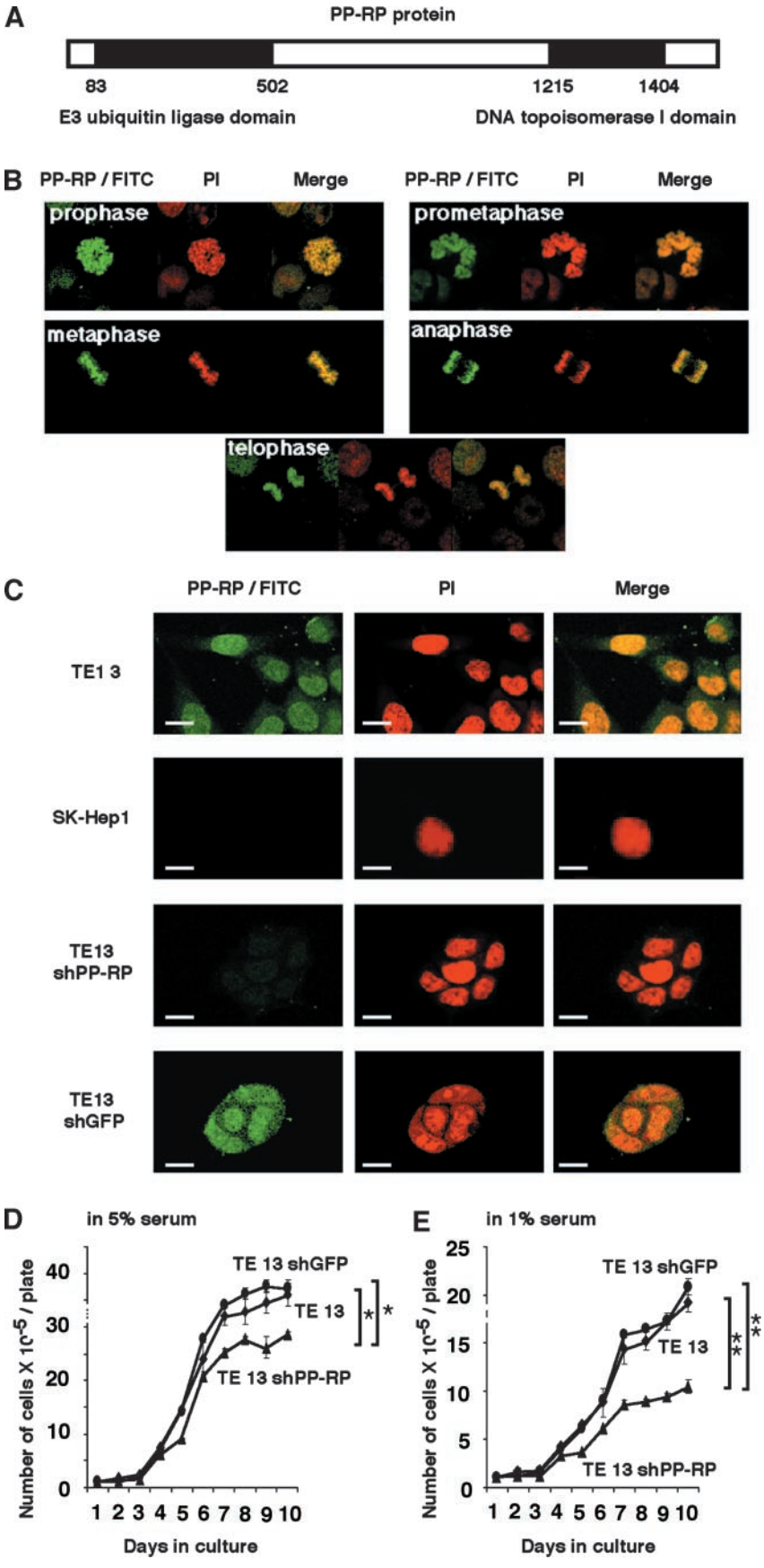

Fig. 9 Reduced proliferation of PP-RP–knockdown TE13 cells and colocalization of PP-RP with chromosomes. *A*, a scheme for domain composition of PP-RP protein. *B*, colocalization of PP-RP with chromosomes. TE13 cells in mitotic phases were fixed and stained with an anti-PP-RP antibody (PP-RP, *green*) 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. ∗, $P < 0.05$; ∗∗, $P < 0.01$.

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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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