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/.

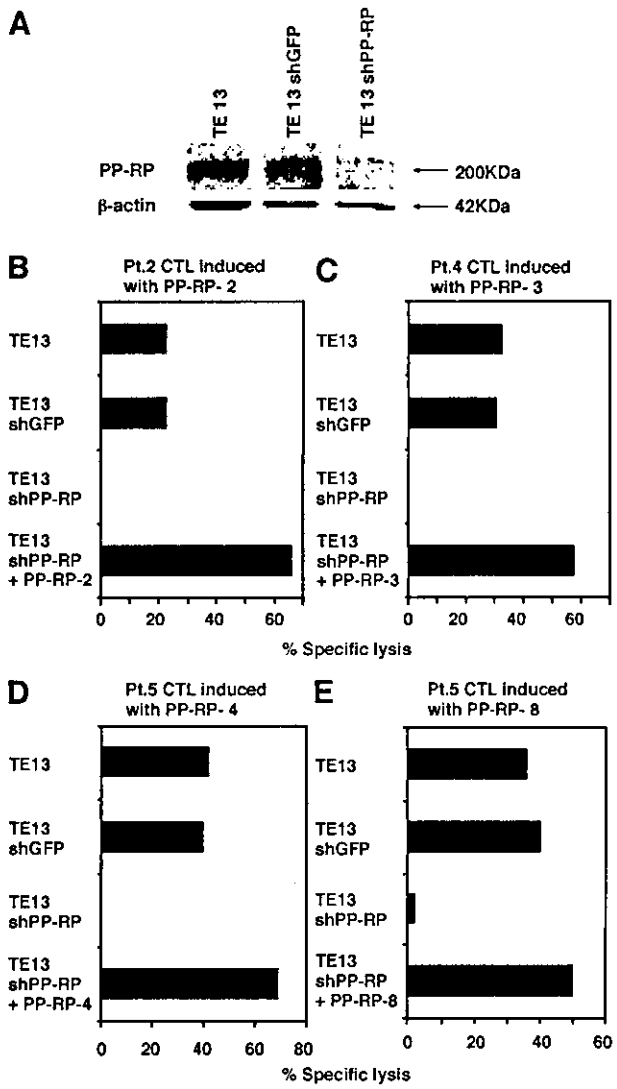

Fig. 7 Abrogation of PP-RP–specific CTL activity by down-regulation of PP-RP protein in TE13 target cells. *A*, down-regulation of PP-RP protein in TE13 cells transfected with pSilencer vector containing the human PP-RP siRNA TE13shPP-RP but not in control cells transfected with pSilencer vector containing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

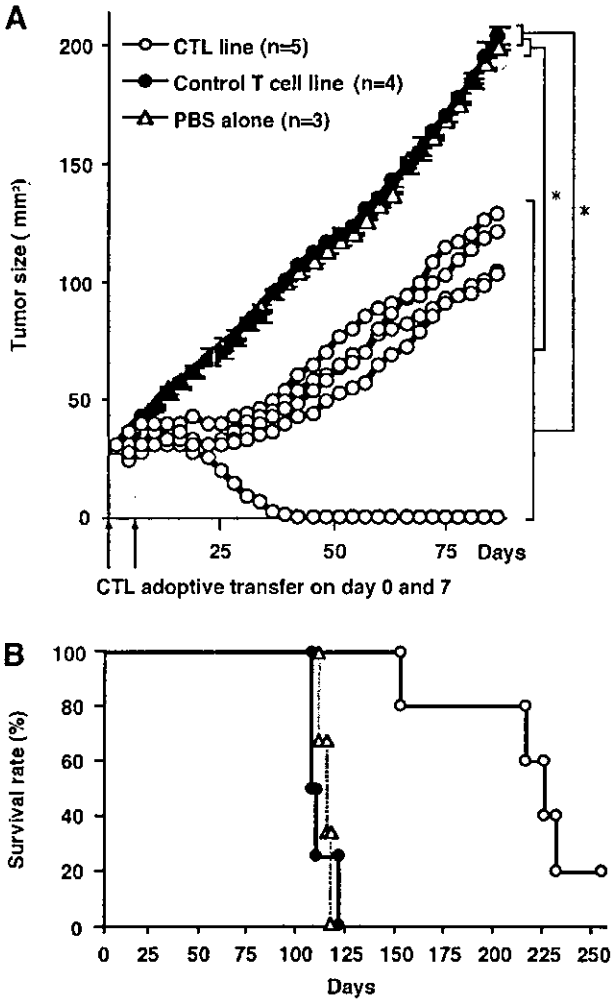

Fig. 8 *In vivo* antitumor activity of adoptively transferred PP-RP-8–induced CTLs. *A*, marked inhibition of growth of a human esophageal cancer cell line, TE11, engrafted into nude mice after adoptive CTL transfer. On day 0, tumor size was 30 mm^2, and CTLs (5×10^6) were inoculated into the tumor.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

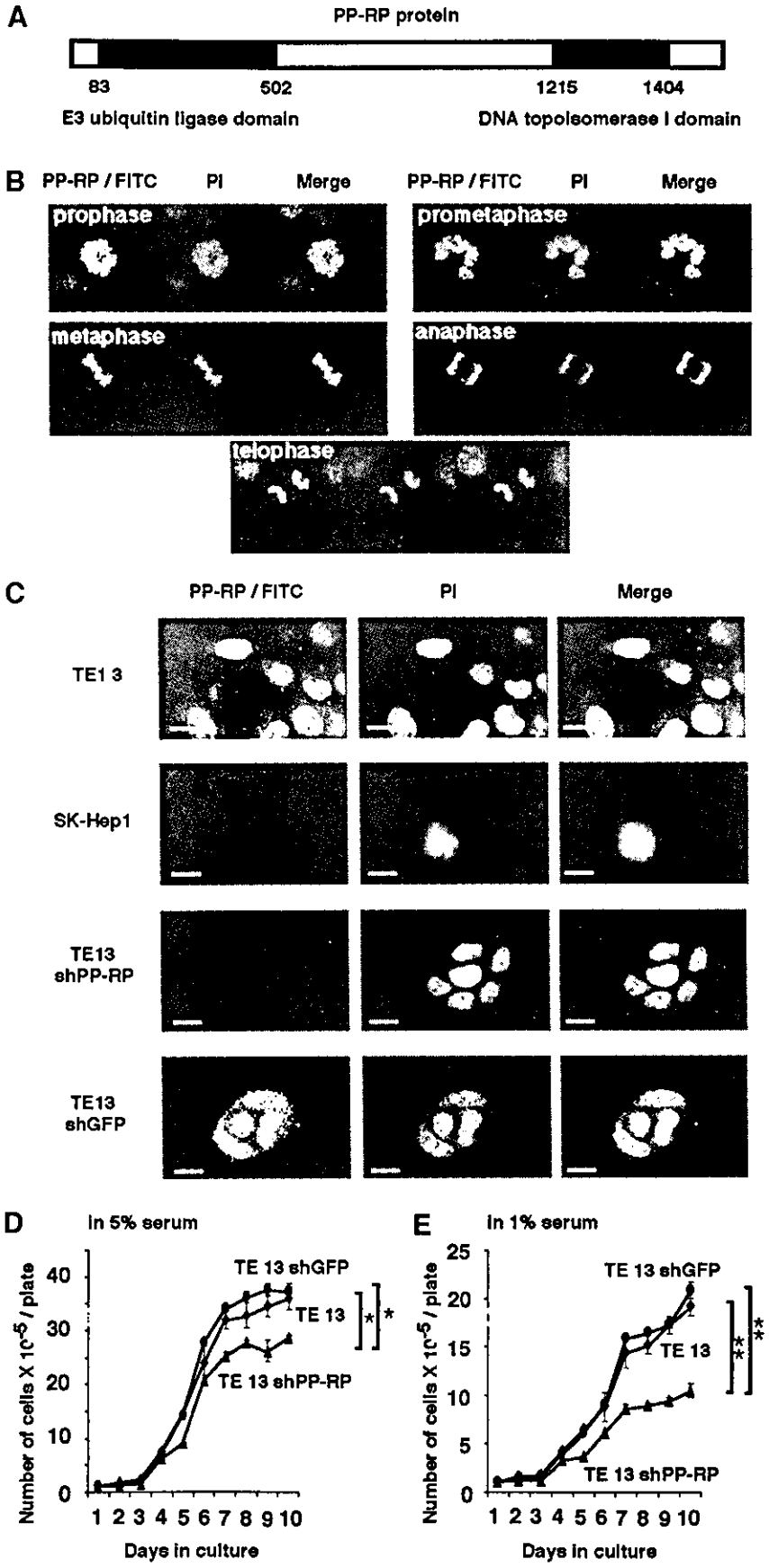

Fig. 9 Reduced proliferation of PP-RP–knockdown TE13 cells and colocalization of PP-RP with chromosomes. *A*, a scheme for domain composition of PP-RP protein. *B*, colocalization of PP-RP with chromosomes. TE13 cells in mitotic phases were fixed and stained with an anti-PP-RP antibody (PP-RP, *green*)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

ACKNOWLEDGMENTS

We thank Drs. Masafumi Takiguchi (Kumamoto University, Kumamoto, Japan), Kyogo Itoh (Kurume University, Kurume, Japan), and the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Sendai, Japan) for providing the cell lines and for the helpful suggestions. We also thank Tatsuko Kubo (Department of Molecular Pathology, Kumamoto University, Kumamoto, Japan) for technical assistance with immunohistochemical analyses and Mariko Ohara (Fukuoka University) for helpful comments.

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

Mikio Monji,[1,4] Tetsuya Nakatsura,[1] Satoru Senju,[1] Yoshihiro Yoshitake,[1,2] Motohiro Sawatsubashi,[4] Masanori Shinohara,[2] Toshiro Kageshita,[3] Tomomichi Ono,[3] Akira Inokuchi,[4] and Yasuharu Nishimura[1]

Departments of [1]Immunogenetics, [2]Oral and Maxillofacial Surgery, and [3]Dermatology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto; and [4]Department of Otolaryngology Head and Neck Surgery, Faculty of Medicine, Saga University, Saga, Japan

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

Received 3/8/04; revised 5/25/04; accepted 6/4/04.

Grant support: Supported in part by Grants-in-Aid 12213111 (Y. Nishimura) and 14770142 (T. Nakatsura) from the Ministry of Education, Science, Technology, Sports and Culture, Japan; by a grant from the Sagawa Science and Technology Promotion Foundation, Japan (Y. Nishimura); and by a grant from Kumamoto Technology and Industry Foundation (Y. Nishimura).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Yasuharu Nishimura, Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan. Phone: 81-96-373-5310; Fax: 81-96-373-5314; E-mail: mxnishim@gpo.kumamoto-u.ac.jp.

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,

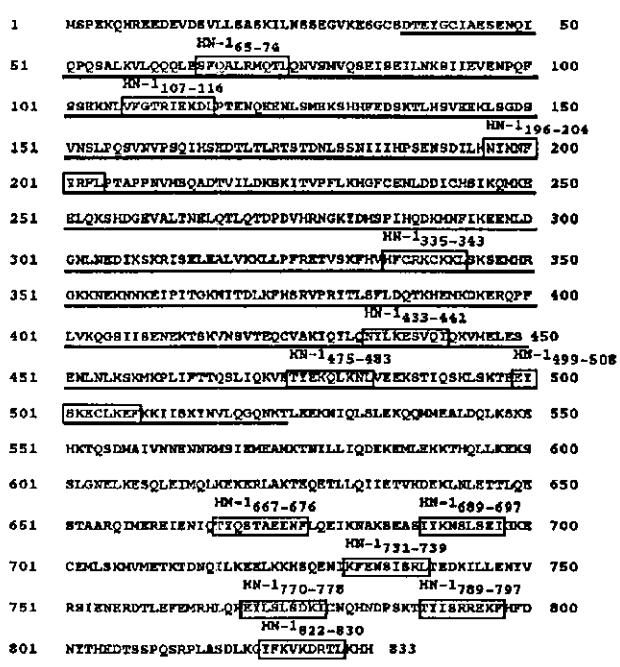

Fig. 1 Predicted amino acid sequences of human KM-HN-1 protein. *Underlined amino acid sequence* corresponds to a recombinant KM-HN-1 protein produced as GST fusion protein; *open box*, synthetic peptides, which have binding motifs for *HLA-A*2402*–encoded molecules, for use of CTL generation.

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.

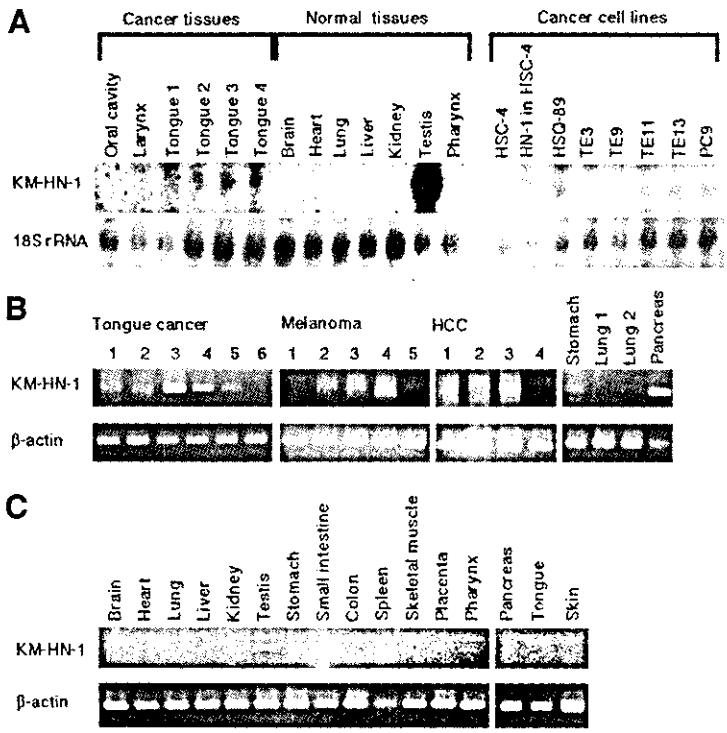

Fig. 2 Expression of *KM-HN-1* mRNA in normal and cancer tissues, and cancer cell lines. *A*, Northern blot analysis of *KM-HN-1* mRNA in various normal and cancer tissues, and cancer cell lines. The same filters were stripped and rehybridized with 18S rRNA to prove RNA integrity and to assess loading 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/GNN*ATG*G) 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/ no. tested‡	*P* value	
Tissue type	Tumor tissues	Cell lines	Age mean	Mean	SD		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.

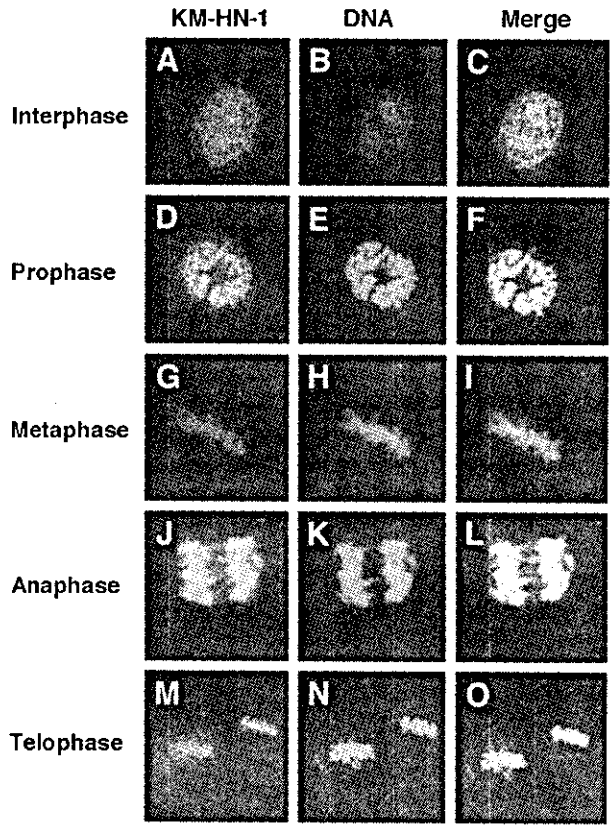

Fig. 3 Immunocytochemical analysis of KM-HN-1 protein with an esophageal cancer cell line, TE13, during mitosis: interphase (*A–C*), prophase (*D–F*), metaphase (*G–I*), anaphase (*J–L*), and telophase (*M–O*). TE13 cells were stained with anti-KM-HN-1 antibody (*green*). Chromosomes were visualized by propidium iodide staining (*red*).

against GST, ELISA reactivity was blocked by prior incubation of sera with an excess amount of purified GST-KM-HN-1 (data not shown). These results confirmed the specificity of IgG to KM-HN-1 in these patients and suggest that KM-HN-1 could elicit humoral immune responses in patients with a variety of tumors.

Association between *KM-HN-1* Expression in Cancer and Presence of KM-HN-1 Antibody in Patients' Sera. Both fresh-frozen tumor specimens and serum samples were available from 13 cancer patients (5 melanomas, 4 HCC, and 4 tongue cancer). Tumors were tested for *KM-HN-1* expression by quantitative RT-PCR, and sera were assayed for KM-HN-1 antibody by ELISA (Table 3). In this series of 13 patients, 5 had both KM-HN-1–positive tumors and KM-HN-1 antibodies. No KM-HN-1 antibody was detected in five patients with KM-HN-1 negative tumors. Three patients had KM-HN-1–positive tumors and no detectable KM-HN-1 antibody. Although the total numbers of samples were small, the association between *KM-HN-1* expression in cancer and presence of KM-HN-1 antibody in patients' sera was statistically significant ($P < 0.05$).

Establishment of CTL Lines Specific to KM-HN-1 Peptide. We attempted to generate KM-HN-1–specific CTLs from four *HLA-A*2402*–positive healthy individuals and four *HLA-A*2402*–positive SCCHN patients, by using KM-HN-1 peptide-pulsed dendritic cell. After three stimulations, CTL activity against peptide-pulsed C1R-A*2402 cells was examined by measuring IFN-γ production to assess peptide specificity. As shown in Fig. 6*A*, CTLs recognizing KM-HN-1 derived peptide were generated from all SCCHN patients and two of four healthy donors. The peptide-reactive CTL lines specific to HN-$1_{196-204}$, HN-$1_{499-508}$, and HN-$1_{770-778}$ were induced from two (SCCHN3 and HD1), three (SCCHN1, SCCHN3, and HD1), and four (SCCHN2, SCCHN4, HD1, and HD2) donors, respectively. The background levels of IFN-γ production in response to peptide unpulsed C1R-A*2402 cells were less than 20 pg/mL. These CTL lines did not produce IFN-γ when exposed to C1R-A*2402 cells pulsed with an HIV-1 nef-derived peptide that had a high binding affinity to HLA-A24, or when exposed to KM-HN-1 peptide-loaded parent cell line, C1R cells, negative for *HLA-A*2402* (data not shown). These data indicate that these CTL activities were KM-HN-1–derived peptide-specific and restricted by *HLA-A*2402* gene product.

To investigate whether 3 KM-HN-1–derived peptides, recognized by CTL lines, are naturally processed and presented by *HLA-A*2402*–encoded molecules, SCCHN cell line HSC-4 (*HLA-A*2402* positive, KM-HN-1 negative) and HSC-4-HN-1, the HSC-4 cells transfected with full-length *KM-HN-1* cDNA, were used as target cells. As shown in Fig. 6*B*, CTL lines induced by HN-$1_{196-204}$, HN-$1_{499-508}$ produced substantial amounts of IFN-γ when exposed to HSC-4-HN-1, but CTL lines induced by HN-$1_{770-778}$ did not do so. The background levels of IFN-γ production by the cells in response to HSC-4 were less than 30 pg/mL in Fig. 6*B*.

Reactivity and Cytotoxicity of CTL Lines against Cancer Cell Lines. 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 and genotyping, and *KM-HN-1* expression was examined by Northern blot analyses. Among the seven cell lines examined, TE9 were negative for HLA-A24, and HSC-4 were negative for KM-HN-1. Because the total amount of blood obtained from cancer patients was limited, we could use CTL lines from cancer patients only for limited experiments. The CTL reactivity was detected when CTLs were exposed to cancer cell lines positive for both HLA-A24 and KM-HN-1. Representative data are shown in Fig. 7*A*, *B*, and *C*. CTL lines induced by HN-$1_{196-204}$, HN-$1_{499-508}$, and HN-$1_{770-778}$ produced substantial amounts of IFN-γ in response to all cancer cell lines positive for both HLA-A24 and KM-HN-1. Cytotoxic activity of these CTL lines was also examined. These CTL lines exhibited cytotoxicity only against TE3 and TE11, which were positive for both HLA-A24 and KM-HN-1, but not against TE9 and HSC-4 (Fig. 8*A–G*).

To further confirm that the cytotoxicity of these CTL lines against cancer cells was mediated by specific recognition of endogenously processed KM-HN-1, we did a cold target inhibition assay as shown in Fig. 8*J*, *K*, and *L*. In the presence of cold target C1R-A*2402 loaded with HN-$1_{196-204}$, HN-$1_{499-508}$, or HN-$1_{770-778}$ peptides, cytotoxic activity of these CTL lines against PC-9 (Fig. 8*J* and *K*) or IFN-γ treated TE13 (Fig. 8 *L*) was markedly inhibited, whereas the addition of HIV-1 nef-derived peptide-loaded or -unloaded C1R-A*2402 had no effect on cytotoxicity. These findings clearly indicate

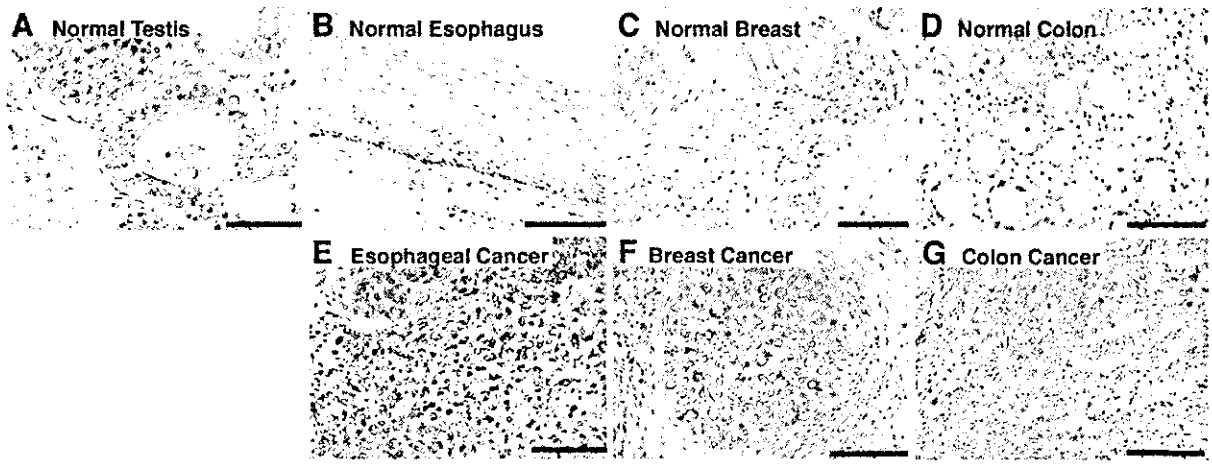

Fig. 4 Immunohistochemical staining of KM-HN-1 antigen in normal and cancer tissues by using avidin-biotin complex method and 3,3′-diaminobenzidine chromogen. *A*. in testis, seminiferous tubules were positively stained with strong intratubular staining of mostly spermatogenic cells, whereas no reactivity with spermatids or with Sertoli cell or interstitial tissue was observed. *B*, *C*, and *D*. KM-HN-1 staining was not detected in the normal esophagus, breast, or colon. *E*. Heterogeneous KM-HN-1 staining was observed in esophageal cancer. Homogeneous KM-HN-1 staining was observed in both breast cancer (*F*) and colon cancer (*G*). ×200. *Scale bar*, 100 μm.

that HN-$1_{196-204}$, HN-$1_{499-508}$, and HN-$1_{770-778}$ peptides are naturally processed from KM-HN-1 protein in cancer cells, expressed in the context of HLA-A24, and recognized by KM-HN-1–specific CTL lines to induce cytotoxicity against cancer cells.

Effects of IFN-γ on Susceptibility of Cancer Cells to CTL-Mediated Cytotoxity. Until now, several antigenic peptides that were processed exclusively by immunoproteasome but not standard proteasome, were reported (31, 32). CTL lines specific to HN-$1_{770-778}$ peptide derived from four different donors were reactive against HN-$1_{770-778}$ peptide-pulsed C1R-A*2402 cells but not against HSC-4-HN-1 (Fig. 6*A* and *B*). We thought that this peptide might be processed by immunoproteasome. Most nonlymphoid cells, be they normal or tumoral, constitutively express standard proteasomes and switch to immunoproteasomes when exposed to

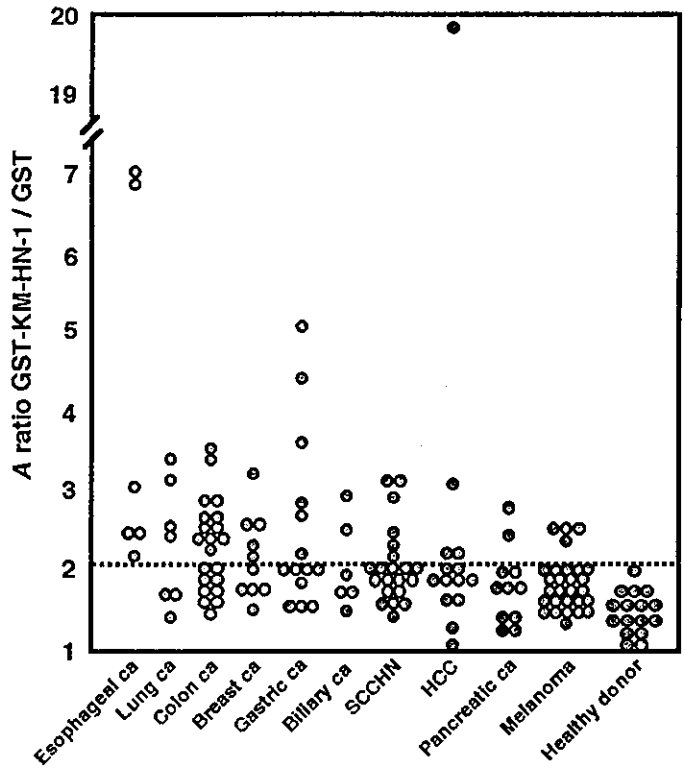

Fig. 5 Quantitation of KM-HN-1– specific IgG measured by ELISA in sera from age-matched healthy donors and patients with various types of cancer. Serum samples were diluted at 1:100 and were analyzed by ELISA to detect antibodies reactive to GST-KM-HN-1 fusion protein and GST alone. The ratio of the absorbance (*A*) for GST-KM-HN-1 to the absorbance for GST was calculated to express the degree of specific reactivity above background. *Dashed line*, the upper normal value (two SDs above the mean value in healthy donors).

Table 3 Correlation between *KM-HN-1* mRNA expression in cancer and the presence of anti-KM-HN-1 antibody in patients' sera

Patients	mRNA*		IgG†	
	Relative ratio		Absorbance ratio	
Melanoma				
1	−	ND	−	1.68
2	+	0.1	+	2.36
3	+	0.5	−	1.71
4	+	0.7	+	2.49
5	−	ND	−	1.87
HCC				
1	−	ND	−	1.99
2	−	ND	−	1.39
3	+	4.0	+	3.46
4	−	ND	−	1.64
Tongue cancer				
1	+	0.5	−	1.39
2	+	1.0	−	1.74
3	+	5.0	+	3.14
4	+	1.0	+	2.56

Abbreviation: ND, not determined.

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

† Antibody titer was determined using indirect ELISA with KM-HN-1-GST fusion protein. The cutoff value (2.15) is the mean plus two SD for healthy donors' sera; positive values are underlined.

IFN-γ (33). Therefore, we investigated effects of IFN-γ on the susceptibility of cancer cells to CTL-mediated cytotoxicity. Target cell lines (HSC-4 and HSC-4-HN-1) were pretreated with IFN-γ according to the protocol described in Materials and Methods. CTL lines specific to HN-$1_{770-778}$ peptide produced IFN-γ in response to IFN-γ treated target cells (Fig. 6*C*). Furthermore, HN-$1_{196-204}$, and HN-$1_{499-508}$ peptide-specific CTL lines also showed increased IFN-γ production in response to IFN-γ-treated target cells. All of CTL lines that were reactive to peptide-pulsed C1R-A*2402 cells produced substantial amounts of IFN-γ in response to HSC-4-HN-1 treated with IFN-γ, but not in response to HSC-4 treated with IFN-γ (data not shown). The background levels of IFN-γ production by the cells in response to HSC-4 were under 50 pg/mL in Fig. 6C. Next, we investigated effects of IFN-γ on the presentation of naturally processed KM-HN-1 peptide in cancer cell lines. Cytotoxic activities of all of these CTL lines tested were also increased when TE13 was pretreated with IFN-γ (Fig. 8*H* and *I*), but not when TE9 and HSC-4 were pretreated with IFN-γ (data not shown).

DISCUSSION

A prerequisite for a broader application of antigen-specific immunotherapy for cancer is the molecular definition of antigens that are specifically expressed in commonly occurring neoplasms, *e.g.*, breast, lung, prostate, or colorectal carcinoma. The recognition that members of the *MAGE, BAGE, GAGE, HOM-MEL-40,* and *NY-ESO-1* gene families form a class of tumor antigens with restricted expression confined to cancer and testis has let to the designation of CT antigens. This characteristic expression profile suggested that testicular tissue is a prime candidate source for the identification of additional CT antigens by SEREX. To further increase the yield of unidentified CT antigens, we applied SEREX with serum from a SCCHN patient and a testis cDNA expression library to identify KM-HN-1.

Although KM-HN-1 contains no Pfam motif (http://pham.wustl.edu/), it does consistently exhibit a low level (<20%) of substantial homology with CENP-F (centromere protein F) and

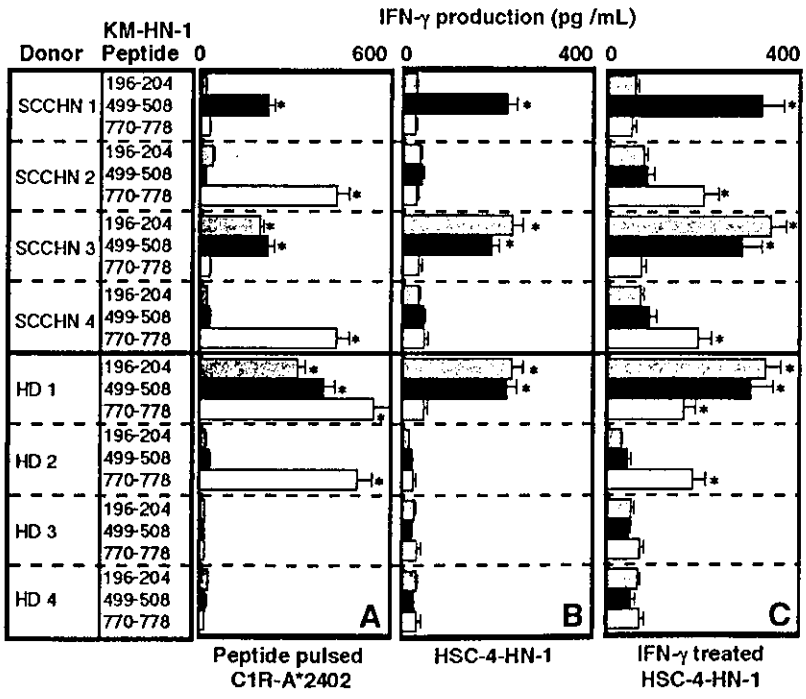

Fig. 6 Induction of CTLs by the KM-HN-1—derived peptides. CTL lines specific to three peptides, HN-$1_{196-204}$, HN-$1_{499-508}$, and HN-$1_{770-778}$, could be generated from HLA-A24–positive healthy donors and cancer patients. CTL responses to the peptide-loaded C1R-A*2402 cells (*A*), HSC-4 cells transfected with *KM-HN-1* gene (expression vector), HSC-4-HN-1 (*B*), and HSC-4-HN-1 pretreated with IFN-γ (*C*) were examined. CTL lines were cultured with each cell for 16 hours, and the culture supernatant was harvested for the measurement of IFN-γ production. *Values*, the means of duplicate assays. Two-tailed Student's *t* test was used for the statistical analysis of difference between IFN-γ production by the cells in response to the corresponding peptide-loaded C1R-A*2402 cells, HSC-4-HN-1, or IFN-γ–treated HSC-4-HN-1, and that in response to unloaded C1R-A*2402 cells, HSC-4, or IFN-γ–pretreated HSC-4, respectively. *, $P < 0.05$. Effector to target ratio was 10. (*HD*, healthy donor.)

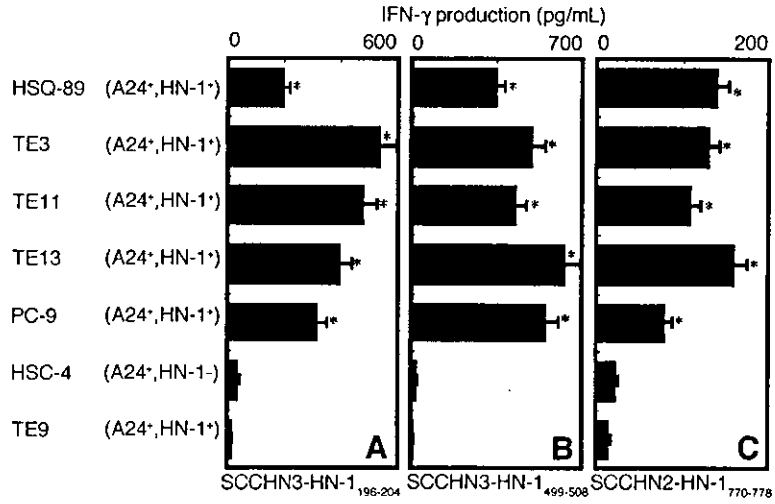

Fig. 7 CTL responses to cancer cell lines. Representative results of at least two reproducible experiments are shown: *A*, HN-$1_{196-204}$ peptide-specific CTL line generated from the patient SCCHN3; *B*, HN-$1_{499-508}$ peptide-specific CTL line generated from the patient SCCHN3; C, HN-$1_{770-778}$ peptide-specific CTL line generated from the patient SCCHN2. CTL lines were cultured with each cancer cell, and the culture supernatant was harvested for the measurement of IFN-γ production. Values represent the means of duplicate assays. Two-tailed Student's *t* test was used for the statistical analysis of difference in IFN-γ production between cancer cell line-stimulated CTLs and HSC-4-stimulated CTLs. *, $P < 0.05$.

SCP-1 (sterol carrier protein-2). CENP-F protein localizes to the spindle midzone and the intracellular bridge in late anaphase and telophase, respectively. Localization of this protein suggests that it may play a role in chromosome segregation during mitosis (34, 35). Furthermore, autoantibodies against CENP-F protein were found in patients with cancer (36). SCP-1 had been described as a major component of the synaptonemal complex (37). The synaptonemal complex is a tripartite macromolecular assembly formed between homologous chromosomes during the meiotic prophase (38). SCP-1 had also been described as one of the CT antigens, recognized by both humoral and cellular immune systems in cancer patients (39). In this study, KM-HN-1 is colocalized with chromosome during mitosis (Fig. 3), and an antibody against KM-HN-1 is observed in cancer patients (Fig. 5 and Table 1), which suggests a functional similarity between KM-HN-1 and these proteins.

Our expression analysis demonstrates the aberrant expression of the *KM-HN-1* gene in a broad spectrum of human neoplasms and characterizes KM-HN-1 as a CT antigen. KM-HN-1 has several similarities to known CT antigens. First, there is the mRNA expression profile; *KM-HN-1* was expressed only in testis among normal tissues but was expressed in various types of cancer, *e.g.*, SCCHN, esophageal cancers, and so forth. Second, there is the protein expression pattern in testis tissues: in the testis, CT antigens expression was detected exclusively in spermatogonia (40). As shown in Fig. 4, our data were similar to the observations in previous studies. Third, KM-HN-1 is immunogenic in cancer patients: KM-HN-1 elicited humoral (Fig. 5) and cellular (Figs. 6, 7, and 8) immune response in cancer patients. On the other hand, KM-HN-1 differs from known CT antigens by chromosomal localization. Although nearly all of the hitherto known genes encoding CT antigens have been mapped to the X chromosome (41), mapping of the human *KM-HN-1* gene has been assigned to chromosome 4. Furthermore, some CT antigens, *e.g.*, MAGE, GAGE, and NY-ESO-1, were shown to be members of multigene families (42, 43), but the family gene of KM-HN-1 was not found.

Because previous studies showed that some of SEREX-defined antigens elicited humoral immune responses in many types of cancer patients (44, 45), we looked for the KM-HN-1-specific antibody in the sera of cancer patients with ELISA methods. Surprisingly an anti-KM-HN-1 IgG was observed in 14 to 100% of all types of cancer patients tested, on the other hand this was not observed in healthy donors (Fig. 5; Table 2). Thereby quantification of anti-KM-HN-1 IgG in sera may provide us a new diagnostic method for various types of cancer. However, a statistically significant association between KM-HN-1 antibody status and clinical characteristics of patients (age, sex, stage) was not evidenced (data not shown).

In this study, we used synthetic KM-HN-1 peptides consisting of HLA-A24 binding motifs for the generation of CTLs from PBMCs, because the *HLA-A24* allele is the most frequent HLA class I allele in the Japanese population and is found in 60% of the Japanese (95% of these cases are genotypically *A*2402*), in 30% of Chinese, and in 20% of Caucasians (46). Of the 13 synthetic peptides used, three peptides (HN-$1_{196-204}$, HN-$1_{499-508}$, and HN-$1_{770-778}$) induced peptide-specific CTL lines. CTLs recognizing KM-HN-derived peptide were generated from all four SCCHN patients and two of four healthy donors. Although the KM-HN-1-specific CTL-precursor frequency of SCCHN patients seemed to be higher than that of healthy donors, peptide-specific CTLs were not detected by intracellular staining of IFN-γ with PBMCs that were stimulated with KM-HN-1 peptides *ex vivo*, in either cancer patients or healthy donors. Peptide-specific CTLs could be detected after two stimulations with antigenic peptide, but a statistically significant difference in frequency of IFN-γ producing CTLs (1–4% of $CD8^+$ T cell) between cancer patients and healthy donors was not observed (data not shown).

Further examination showed that CTL lines specific to only two (HN-$1_{196-204}$ and HN-$1_{499-508}$) of these peptides lysed *KM-HN-1* transfectant (HSC-4-HN-1), but all CTL lines reactive to peptide-pulsed C1R-A*2402 lysed HSC-4-HN-1 pretreated with IFN-γ (Fig. 6). Similar results are shown for CTL activities against cancer cell lines (Fig. 8). When cancer cell lines were treated with IFN-γ, the *KM-HN-1* mRNA level did not change (data not shown). In general, IFN-γ induces several changes in cancer cells, *e.g.*, up-regulation of MHC class I

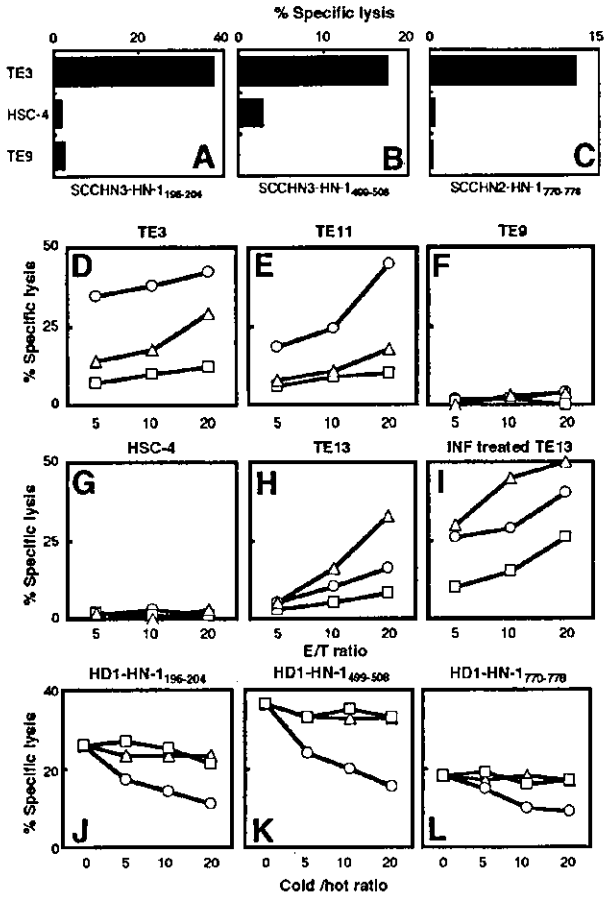

Fig. 8 ^{51}Cr release assay for cytotoxicity of CTL lines against cancer cell lines. *A–C*, cytotoxic activities of CTL lines generated from SCCHN patients against cancer cell line were detected by 4-hour ^{51}Cr release assay at effector-to-target ratio of 10:1. Peptides used for CTL generation were indicated in abscissa (*D–I*), HN-$1_{196-204}$ ([trio]), HN-$1_{499-508}$ (○), and HN-$1_{770-778}$ (□) peptide-specific CTL lines generated from healthy donor (*HD*) 1 were used as effector cells, respectively. Cytotoxic activities against TE3 (*D*) and TE11 (*E*) expressing both HLA-A24 and KM-HN-1, TE9 (KM-HN-1$^+$ and HLA-A24$^-$; *F*) and HSC-4 (KM-HN-1$^-$ and HLA-A24$^+$; *G*). Cytotoxic activities against TE13, expressing both HLA-A24 and KM-HN-1 (*H*) and TE13 pretreated with IFN-γ (*I*). *J–L*, cold target inhibition assay. ^{51}Cr-labeled PC-9 (*J* and *K*) and TE13 (*L*) cells pretreated with IFN-γ were mixed with various numbers of unlabeled C1R-A*2402 cells that had been unloaded (△) or loaded with the KM-HN-1 peptide indicated (○) or with irrelevant HIV peptide (□). The cytotoxicity of the ^{51}Cr-labeled target cells in the presence of unlabeled target cells was determined by 4-hour ^{51}Cr release assays at an effector-to-^{51}Cr-labeled-target-cell ratio of 10:1.

molecules and induction of expression of the immunoproteasome (47). Our results may be attributable to both of these mechanisms. First, after cancer cell lines used in this study were treated with IFN-γ, the expression of HLA-A24 molecules increased two (HSC-4-HN-1) and five (TE13) times (data not shown). Second, although HLA expression increased 2- and 5-fold, CTL lines specific to HN-$1_{770-778}$ showed an almost 10-fold increase of CTL activities when exposed to IFN-γ-treated target cells.

Identification of new CTL epitopes in different tumor antigens will allow for development of multiantigenic (epitope-based) tumor vaccines, which will probably be useful to circumvent tumor escape from immune systems by losing expression of antigen. In this study, we found a novel human cancer/testis antigen, KM-HN-1. *KM-HN-1* gene is expressed in many types of cancer, and a humoral immune response to KM-HN-1 protein is detected in many cancer patients. Furthermore, three peptides derived from KM-HN-1 could induce HLA-A24-restricted and tumor-reactive CTL lines. These results suggest that KM-HN-1 might be a good candidate for the development of a cancer vaccine applicable to various types of cancer patients.

ACKNOWLEDGMENTS

We thank Drs. M. Takiguchi (Kumamoto University, Kumamoto, Japan) and K. Itoh (Kurume University, Kurume, Japan), and the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) for providing tumor samples, cell lines and helpful suggestions. We also thank Dr. T. Yamamoto and T. Kubo (Department of Molecular Pathology, Kumamoto University) for technical assistance with the immunohistochemical analyses, and M. Ohara (Fukuoka) for helpful comments.

REFERENCES

1. Rosenberg SA. Development of cancer immunotherapies based on identification of the genes encoding cancer regression antigens. J Natl Cancer Inst (Bethesda) 1996;88:1635–44.

2. van der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science (Wash DC)1991;254:1643–7.

3. Traversari C, van der Bruggen P, Van den Eynde B, et al. Transfection and expression of a gene coding for a human melanoma antigen recognized by autologous cytolytic T lymphocytes. Immunogenetics 1992;35:145–52.

4. De Plaen E, Arden K, Traversari C, et al. Structure, chromosomal localization, and expression of 12 genes of the MAGE family. Immunogenetics 1994;40:360–9.

5. Jassim A, Ollier W, Payne A, Biro A, Oliver RT, Festenstein H. Analysis of HLA antigens on germ cells in human semen. Eur J Immunol 1989;19:1215–20.

6. Stockert E, Jager E, Chen YT, et al. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. J Exp Med 1998;187:1349–54.

7. Old LJ. Cancer/testis (CT) antigens—a new link between gametogenesis and cancer. Cancer Immun 2001;1:1.

8. Renkvist N, Castelli C, Robbins PF, Parmiani G. A listing of human tumor antigens recognized by T cells. Cancer Immunol Immunother 2001;50:3–15.

9. Sahin U, Tureci O, Schmitt H, et al. Human neoplasms elicit multiple specific immune responses in the autologous host. Proc Natl Acad Sci USA 1995;92:11810–3.

10. Brichard V, Van Pel A, Wolfel T, et al. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J Exp Med 1993;178:489–95.

11. Gure AO, Tureci O, Sahin U, et al. SSX: a multigene family with several members transcribed in normal testis and human cancer. Int J Cancer 1997;72:965–71.

12. Chen YT, Gure AO, Tsang S, et al. Identification of multiple cancer/testis antigens by allogeneic antibody screening of a melanoma cell line library. Proc Natl Acad Sci USA 1998;95:6919–23.

13. Eichmuller S, Usener D, Dummer R, Stein A, Thiel D, Schadendorf D. Serological detection of cutaneous T-cell lymphoma-associated antigens. Proc Natl Acad Sci USA 2001;98:629–34.

14. 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.

15. Shichijo S, Nakao M, Imai Y, et al. A gene encoding antigenic peptides of human squamous cell carcinoma recognized by cytotoxic T lymphocytes. J Exp Med 1998;187:277–88.

16. 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.

17. Eura M, Chikamatsu K, Katsura F, et al. A wild-type sequence p53 peptide presented by HLA-A24 induces cytotoxic T lymphocytes that recognize squamous cell carcinomas of the head and neck. Clin Cancer Res 2000;6:979–86.

18. 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.

19. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.

20. 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.

21. Yamada K, Senju S, Nakatsura T, et al. Identification of a novel autoantigen UACA in patients with panuveitis. Biochem Biophys Res Commun 2001;280:1169–76.

22. Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene (Amst) 1991;108:193–9.

23. 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.

24. Senju S, Iyama K, Kudo H, Aizawa S, Nishimura Y. Immunocytochemical analyses and targeted gene disruption of GTPBP1. Mol Cell Biol 2000;20:6195–200.

25. 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.

26. 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.

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

28. Kawano K, Gomi S, Tanaka K, et al. Identification of a new endoplasmic reticulum-resident protein recognized by HLA-A24-restricted tumor-infiltrating lymphocytes of lung cancer. Cancer Res 2000;60:3550–8.

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

30. Kozak M. Interpreting cDNA sequences: some insights from studies on translation. Mamm Genome 1996;7:563–74.

31. Schultz ES, Chapiro J, Lurquin C, et al. The production of a new MAGE-3 peptide presented to cytolytic T lymphocytes by HLA-B40 requires the immunoproteasome. J Exp Med 2002;195:391–9.

32. van Hall T, Sijts A, Camps M, et al. Differential influence on cytotoxic T lymphocyte epitope presentation by controlled expression of either proteasome immunosubunits or PA28. J Exp Med 2000;192:483–94.

33. Rock KL, Goldberg AL. Degradation of cell proteins and the generation of MHC class I-presented peptides. Annu Rev Immunol 1999;17:739–79.

34. Liao H, Winkfein RJ, Mack G, Rattner JB, Yen TJ. CENP-F is a protein of the nuclear matrix that assembles onto kinetochores at late G2 and is rapidly degraded after mitosis. J Cell Biol 1995;130:507–18.

35. Hussein D, Taylor SS. Farnesylation of Cenp-F is required for G2/M progression and degradation after mitosis. J Cell Sci 2002;115:3403–14.

36. Rattner JB, Rees J, Whitehead CM, et al. High frequency of neoplasia in patients with autoantibodies to centromere protein CENP-F. Clin Investig Med 1997;20:308–19.

37. Meuwissen RL, Offenberg HH, Dietrich AJ, Riesewijk A, van Iersel M, Heyting C. A coiled-coil related protein specific for synapsed regions of meiotic prophase chromosomes. EMBO J 1992;11:5091–100.

38. von Wettstein D, Rasmussen SW, Holm PB. The synaptonemal complex in genetic segregation. Annu Rev Genet 1984;18:331–413.

39. Tureci O, Sahin U, Zwick C, Koslowski M, Seitz G, Pfreundschuh M. Identification of a meiosis-specific protein as a member of the class of cancer/testis antigens. Proc Natl Acad Sci USA 1998;95:5211–6.

40. Juretic A, Spagnoli GC, Schultz-Thater E, Sarcevic B. Cancer/testis tumour-associated antigens: immunohistochemical detection with monoclonal antibodies. Lancet Oncol 2003;4:104–9.

41. Scanlan MJ, Gure AO, Jungbluth AA, Old LJ, Chen YT. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. Immunol Rev 2002;188:22–32.

42. De Backer O, Arden KC, Boretti M, et al. Characterization of the GAGE genes that are expressed in various human cancers and in normal testis. Cancer Res 1999;59:3157–65.

43. Chen YT, Boyer AD, Viars CS, Tsang S, Old LJ, Arden KC. Genomic cloning and localization of CTAG, a gene encoding an autoimmunogenic cancer-testis antigen NY-ESO-1, to human chromosome Xq28. Cytogenet Cell Genet 1997;79:237–40.

44. Yang XF, Wu CJ, Chen L, et al. CML28 is a broadly immunogenic antigen, which is overexpressed in tumor cells. Cancer Res 2002;62:5517–22.

45. Yang XF, Wu CJ, McLaughlin S, et al. CML66, a broadly immunogenic tumor antigen, elicits a humoral immune response associated with remission of chronic myelogenous leukemia. Proc Natl Acad Sci USA 2001;98:7492–7.

46. Imanishi AT, Kimura A, Tokunaga K, Gojobori T. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In: Tsuji K, Aizawa M, Sasasuki T, editors. Proceedings of the Eleventh International Histocompatibility Workshop and Conference. Oxford: Oxford University Press; 1992. p. 1065–220.

47. Fruh K, Yang Y. Antigen presentation by MHC class I and its regulation by interferon gamma. Curr Opin Immunol 1999;11:76–81.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 321 (2004) 432–440
BBRC
www.elsevier.com/locate/ybbrc

Detection of the novel autoantibody (anti-UACA antibody) in patients with Graves' disease

Tsuyoshi Ohkura [a], Shin-ichi Taniguchi [a,*], Kazuhiro Yamada [b], Naoko Nishio [a], Tomohisa Okamura [a], Akio Yoshida [a], Keiichi Kamijou [c], Shuji Fukata [d], Kanji Kuma [d], Yoichi Inoue [e], Ichiro Hisatome [f], Satoru Senju [g], Yasuharu Nishimura [g], Chiaki Shigemasa [a]

[a] *Division of Molecular Medicine and Therapeutics, Department of Multidisciplinary Internal Medicine, Tottori University Faculty of Medicine, Yonago 683-8504, Japan*
[b] *Department of Ophthalmology and Visual Science, Kumamoto University Graduate School of Medical Sciences, Kumamoto 860-0811, Japan*
[c] *Kamijou Thyroid Research Institute, Sapporo 060-0042, Japan*
[d] *Kuma Hospital, Kobe 650-0011, Japan*
[e] *Olympia Eye Hospital, Tokyo 150-0001, Japan*
[f] *School of Life Science, Faculty of Medicine, Tottori University, Yonago 683-8504, Japan*
[g] *Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Science, Kumamoto 860-0811, Japan*

Received 9 June 2004

Abstract

Uveal autoantigen with coiled coil domains and ankyrin repeats (UACA) is an autoantigen in patients with panuveitis such as Vogt–Koyanagi–Harada disease. The prevalence of IgG anti-UACA antibodies in patients with uveitis is significantly higher than healthy controls, suggesting its potential role as an autoantigen. Originally, UACA was cloned from dog thyroid tissue following TSH stimulation. So, we presumed UACA could be a novel autoantigen in autoimmune thyroid diseases. We measured serum anti-UACA antibody titer using ELISA in patients with autoimmune thyroid diseases (Graves' disease, Hashimoto's thyroiditis, subacute thyroiditis, and silent thyroiditis). The prevalence of anti-UACA antibodies in Graves' disease group was significantly higher than that in healthy group (15% vs. 0%). Moreover, the prevalence of anti-UACA antibodies in Graves' ophthalmopathy was significantly higher than that in Graves' patients without ophthalmopathy (29% vs. 11%). Especially, 75% of severe ocular myopathy cases showed high UACA titer. Immunohistochemical analysis revealed that UACA protein is expressed in eye muscles as well as human thyroid follicular cells. Taken together, UACA is a novel candidate for eye muscle autoantigens in thyroid-associated ophthalmopathy.

Keywords: Uveal autoantigen with coiled coil domains and ankyrin repeats; Graves' disease; Graves' ophthalmopathy; Autoantigen; Ocular myopathy; FRTL5; Vogt–Koyanagi–Harada disease; Thyroid–eye shared autoantigen

Uveal autoantigen with coiled coil domains and ankyrin repeats (UACA) is an autoantigen associated with panuveitis. Anti-UACA antibody appears in patients sera of Vogt–Koyanagi–Harada disease (VKH), sarcoidosis, and Behçet disease with uveitis. Although UACA is expressed in various tissues such as skeletal muscle and melanocyte, the appearance of anti-UACA antibody seems to reflect the autoimmune reaction against uveal melanocyte [1]. Interestingly, UACA was originally

* Corresponding author. Fax: +81-859-34-8099.
E-mail address: stani@grape.med.tottori-u.ac.jp (S. Taniguchi).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.06.162

identified from dog thyroid as one of TSH regulated genes with unknown function [2]. The dual expression of UACA in thyroid and skeletal muscle led us to the idea that UACA could be an autoantigen associated with Graves' disease, since patients with Graves' disease frequently suffer from ophthalmopathy with ocular myopathy [3–15].

Thyroid-associated ophthalmopathy (TAO) is considered to be an autoimmune disorder of eye muscle and surrounding orbital connective tissue and fat, and the current dogma tells that TAO is induced by autoimmune reaction against thyroid and orbital tissue shared antigens [3–21]. One such candidate is TSH receptor, which is expressed in the orbital preadipocyte and fibroblast [8–11]. Several eye muscle and thyroid shared antigens also have clinical relevance in TAO; flavoprotein [16,17], 1D [18], and G2s protein [22–24]. The primary reaction in ocular tissue is thought to be T-cell-mediated autoimmunity against TSH receptor (TSHR) expressed in ocular fibroblasts. The appearance of antibodies against Fp, G2s, and 1D seems to be the secondary event in TAO process, reflecting the release of sequestered cytoskeletal proteins from damaged eye muscles [23,24]. These eye muscle proteins are expressed in eye muscles as well as skeletal muscles. Since UACA is expressed in skeletal muscle as well as thyroid tissue, we presumed that the appearance of anti-UACA autoantibody could be linked to the autoimmune response associated with Graves' ophthalmopathy.

In this study, we measured serum UACA antibody titer in patients with autoimmune thyroid diseases; healthy controls, Graves' disease, Hashimoto's disease, silent thyroiditis, and subacute thyroiditis. The mean value of UACA antibody titer in the Graves' disease group was significantly higher than healthy controls, but other group was not. Moreover, high UACA titer was observed in Graves' ophthalmopathy patients with severe ocular myopathy.

This is the first report describing the presence of anti-UACA autoantibodies in patients with Graves' disease. Especially, high UACA titer appears to be associated with eye muscle damage of Graves' ophthalmopathy.

Materials and methods

Study patients. We studied 159 Graves' disease, 26 Hashimoto's thyroiditis, 20 silent thyroiditis, 11 subacute thyroiditis, and 43 controls. We explained the purpose of this study to all subjects and obtained their informed consent. Graves' disease patients consisted of untreated 122 females and 37 males. Diagnosis of Graves' disease was confirmed by elevated free T_3 level (13.61 ± 6.67 ng/dl), undetectable TSH level, and positive TSH binding inhibitory immunoglobulin and/or thyroid stimulating antibody. They had hyperthyroidism symptoms such as palpitation and body weight loss. Silent thyroiditis group consisted of 18 females and 2 males with a mean age of 38 year. Diagnosis of silent thyroiditis was confirmed by elevation of free T_3 levels (7.51 ± 2.39 ng/dl), suppressed ^{123}I uptake, and elevated thyroglobulin level. Subacute thyroiditis patients had neck pain and tenderness. They had elevated free T_3 level (8.15 ± 5.48 ng/dl), CRP and ESR level, and suppressed TSH level. Hashimoto's thyroiditis group had elevated TSH levels (69.1 ± 38.2 μU/ml) and positive thyroid TGAb (antithyroglobulin antibody). Forty three normal individuals of similar age and gender were used as controls.

Graves' group included 31 patients with ophthalmopathy (24 females and 8 males, 21–58 years old), 128 patients without ophthalmopathy. The eye changes were classified according to an activity index (AI, 0–7) proposed by a committee of the International Thyroid Associations. Patients with ophthalmopathy were defined as >AI, and patients without ophthalmopathy were defined as A0. Ophthalmologic examination, including measurement of eye muscle function and performance of orbital MRI, was carried out on patients with ophthalmopathy. The congestive changes were defined as >AI, with or without eye muscle involvement. Ocular myopathy was defined as: diplopia and reduced eye movement associated with marked increase of eye muscle volume on orbital MRI. Congestive ophthalmopathy was defined as: nil or minimal eye muscle enlargement with, usually, a fibrotic appearance, as described by Ossoinig [25], features of periorbital inflammation (e.g., chemosis, lid swelling, and conjunctival injection), and no diplopia or reduced eye movements.

Human subjects. Human thyroid tissues were obtained by the University of Tottori committee for the protection of human subjects and in accordance with the Declaration of Helsinki. Thyroid tissue sample was obtained at surgery from a Graves' disease patient. Normal thyroid tissue was obtained at autopsy from a patient without thyroid disease. Human eye muscle tissue with Graves' ophthalmopathy was obtained at surgery from a Graves' disease patient (kindly provided by Dr. Yoichi Inoue, Olympia Eye Hospital).

Preparation of glutathione-S transferase fusion protein. A 783-bp DNA fragment digested from *Homo sapiens* cDNA clone IMAGE 608930 (Embank Accession No. AA197064) corresponding to nucleotide position 3462–4245 of UACA cDNA was inserted into pGEX4T-2 vector to produce glutathione-*S* transferase (GST) UACA fusion protein. This UACA fragment covers C-terminal 261 amino acids (18.0%) of whole UACA consisting of 1449 amino acids. Plasmids with this construct were transformed in *Escherichia coli* and incubated in 500 ml Luria broth medium for 8 h at 37 °C with shaking. Then, IPTG was added at a final concentration of 0.1 mM and the preparation was incubated for 16 h at 25 °C with shaking. This suspension was centrifuged and the pellet was suspended in 20 ml lysis buffer [50 mM Tris–HCl (pH 7.5), 25% sucrose]. Then, we added 100 μl of 10% Nonidet P-40, 1 M $MgCl_2$ on ice. The lysate was sonicated, centrifuged, and then the supernatant was incubated with 2 ml of slurry of glutathione–Sepharose 4B for 2 h at 4 °C. This suspension was centrifuged and the pellet was washed in WE buffer [20 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, and 1 mM DTT] 10 times. The fusion protein was eluted with G buffer [5 mM GSH, 50 mM Tris–HCl (pH 9.6)] and eluted protein concentration was estimated by Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA).

Enzyme-linked immunosorbent assay. Detection and titration of antibody to a fragment of UACA were examined using indirect enzyme-linked immunosorbent assay (ELISA). GST–UACA fusion protein and GST protein were prepared and used as antigens. Microtiter plates (96-well) (NUNC, Denmark) were coated with GST–UACA fusion protein in PBS (pH 7.4) for 15 h at 4 °C. GST protein was simultaneously coated in different wells as control. The plates were then washed with 5% skim milk/PBS for 2 h at room temperature. The plates were washed with PBS-T and incubated for 15 h at 4 °C with serum samples diluted at 1:50 with 1% skim milk/PBS. The plates were washed in PBS-T, and 100 μl of HRP-conjugated mouse anti-human IgG diluted at 1:2000 with 1% skim milk/PBS was added to each well followed by incubation at room temperature for 2 h. The plates were washed with PBS-T, and 100 μl solution of *o*-phenylenediamine (Sigma Fast; Sigma Chemical, St. Louis, MO) was added to each well. After

30 min, the reaction was stopped by adding 50 μl of 3 M H_2SO_4, and OD 490 nm was determined using a Model 550 microplate reader (Bio-Rad, Hercules, CA). The specific corrected OD value of an individual sample was calculated by subtracting the OD value of GST protein coated well from that of GST–UACA fusion protein.

Cell culture. FRTL-5 rat thyroid cells (Interthyr Research Foundation, Baltimore, MD; ATCC No. CRL 8305) were a fresh subclone (F1) that had all properties previously detailed. All cells were grown in 6H medium consisting of Coon's modified F12 (Sigma Chemical, St. Louis, MO) supplemented with 5% calf serum, 1 mM non-essential amino acids (Gibco, Grand Island, NY), and a mixture of six hormones:bovine TSH (1×10^{-10} M), insulin (10 μg/ml), cortisol (0.4 ng/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). Fresh medium was replaced every 2 or 3 days, and cells were passaged every 7–10 days. In different experiments, cells were maintained in 5H medium without TSH and then exposed to TSH for appropriate time period (0, 3, 6, 12, and 24 h). In dose course analysis, FRTL5 cells were incubated with various concentrations (0, 10^{-3}, 10^{-2}, 10^{-1}, and 1 mU/ml) of TSH for 24 h.

The following human thyroid cancer cell lines were obtained from Dr S. Kosugi (Department of Laboratory Medicine and Clinical Genetics Unit, Kyoto University School of Medicine); NPA [26] and FRO [27] thyroid cancer cell lines were grown in RPMI medium 1640 (31800-022, Gibco-BRL, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 50 μg/ml streptomycin. FRO cells, derived from a poorly differentiated follicular thyroid carcinoma, were characterized by the presence of wild-type p53 alleles for exons 5–8 [24]. 8505C [28] and HTC [29] thyroid cancer cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (12800-017, Gibco-BRL, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 50 μg/ml streptomycin. Culture medium was changed every 2 days and cells were passaged every 5–6 days.

Western blot analysis. Cells were lysed on ice in 0.6 ml lysate mix containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride in PBS. For immunoblotting, 10 μg of each sample was electrically transferred to Immunobilon PVDF (polyvinylidene difluoride) Transfer Membranes (Millipore, Bedford, MA). Membranes were incubated in blocking buffer; Tris-buffered saline (TBS; Tris–HCl 10 mM, pH 8.0, and NaCl 150 mM) containing 0.05% [vol/vol] Tween 20 and 5% [wt/vol] non-fat dried milk for overnight. Membranes were then incubated in blocking buffer with rabbit polyclonal anti-UACA antibody (kindly provided by Dr. K. Yamada) (1:500 dilution) or goat polyclonal anti-actin antibody (sc-1616, Santa Cruz Biotechnology, USA) for 45 min, and then washed twice with TBS containing 0.05% Tween 20. Membranes were incubated in blocking buffer with horseradish peroxidase-conjugated anti-rabbit IgG antibody for UACA or horseradish peroxidase-conjugated anti-goat IgG antibody (Amersham, UK) for actin, respectively, washed three times with TBS with 0.05% Tween 20, and then detected with enhanced chemiluminescence reagents (Amersham, UK).

Reverse transcription-PCR. Poly(A)$^+$ RNA were purified from 10 μg of each total RNA and subjected to cDNA synthesis, using random primers and Superscript reverse transcriptase. Gene-specific PCR primers were designed to amplify fragments of 505 bp and used in the reverse transcription-PCR (RT-PCR) (94 °C 30 s, 56 °C 30 s, and 74 °C 4 min, 30 cycles). Forward and reverse primer sequences for PCR amplification of UACA were 5′-GAGAAAAGAAGTTGGAATCAT AA-3′ and 5′-TTGTGTAGGTGAGTTGGGAAAG-3′, respectively.

Immunohistochemical evaluation of UACA expression. UACA expression was analyzed by immunocytochemical staining of Graves' thyroid tissues, eye muscle tissue obtained from a patient with Graves' ophthalmopathy. We immunostained the ocular tissue including extraocular muscles. Paraffin-embedded tissue section, 4-μm thick, was deparaffinized in xylene, rehydrated through a graded alcohol series to deionized water. The endogenous peroxidase activity was blocked with H_2O_2. The tissue section was incubated with rabbit polyclonal anti-UACA antibody (1:10) for 12 h at 4 °C, then washed and incubated with biotinylated horse anti-rabbit IgG (1:3000) for 30 min at room temperature. The sections were immersed in a solution with the avidin–biotin complex (Vector Laboratories, USA) for 30 min, developed with diaminobenzidine, and counterstained with eosin. The sections were scanned at magnification (200×, 400×) using light microscopy. Normal thyroid sample was obtained at autopsy from a patient without thyroid disease.

Immunofluorescent staining and microscopy. FRTL5 cells were plated on coverslips and cultured in Coon's modification HamF 12 with 5% fetal calf serum, then washed twice with PBS, and fixed with 2% paraformaldehyde. Cells were permeabilized with 0.5% Triton X-100, incubated with rabbit polyclonal anti-UACA antibody (1:100), and then visualized using FITC-conjugated anti-rabbit IgG antibody. In order to observe the fine localization of UACA protein within cells, we used confocal microscopy system (FLUOVIEW-OLYMPUS).

Statistical analysis. We used the χ^2 test (with Yeasts' correction for small numbers) and Fisher's exact test for categorical comparisons of the data. Differences in the means of continuous measurements were statistically analyzed using ANOVA. P value of <0.05 was considered to indicate statistical significance. All statistical analyses were performed on a personal computer with the statistical package StatView 5.0 for Macintosh (SAS Institute, Cary, NC).

Results

ELISA

We measured serum UACA antibody titer in patients with autoimmune thyroid diseases in ELISA, using recombinant C-terminal 18% fragment of human UACA protein. We measured titer of healthy controls (43 cases), Graves' disease (159 cases), Hashimoto's disease (26 cases), silent thyroiditis (20 cases), and subacute thyroiditis (11 cases). To exclude the effect of reactivity against GST protein, we used GST–UACA fusion protein and GST protein for ELISA, simultaneously. Evaluation of IgG anti-UACA autoantibodies was determined by subtracting the reactivities against GST from those against GST–UACA. The mean OD value of anti-UACA autoantibodies in Graves' patients was significantly higher than that in healthy controls (ANOVA; $P < 0.01$) (Fig. 1A). This group patient did not accompany VKH disease or other uveitis. In contrast, Hashimoto's thyroiditis, silent thyroiditis, and subacute thyroiditis group did not show any statistical significance compared with control. The cutoff OD value for positivity of anti-UACA IgG antibodies was defined as the mean value +3 SD of healthy controls (0.53). We found anti-UACA IgG antibodies in 15% (24/159) of Graves' patients and 0% (0/43) of healthy control (Table 1). The prevalence of IgG anti-UACA antibodies in Graves' patients was significantly higher than that in healthy control (Fisher's exact test; $P < 0.05$). Anti-UACA antibodies were found in 4% (1/26) of Hashimoto's thyroiditis, 5% (1/20) of silent thyroiditis, and 8% (1/11) of subacute thyroiditis group. The differences in prevalence of anti-UACA antibodies