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

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′-CCATCCCAGATA-CATTCCGAGGAC-3′ and antisense 5′-GGTCGAGGAAG-GACAGTGTGT-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′-CCATCCCAGATACAT-TCCGAGGAC-3′ and antisense 5′-CTGATTTCCGACTG-

TACCATGCTG-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):

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

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, a 1466-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 $A_{GST-KM-HN-1}$ 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 $A_{GST-KM-HN-1}$ 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.
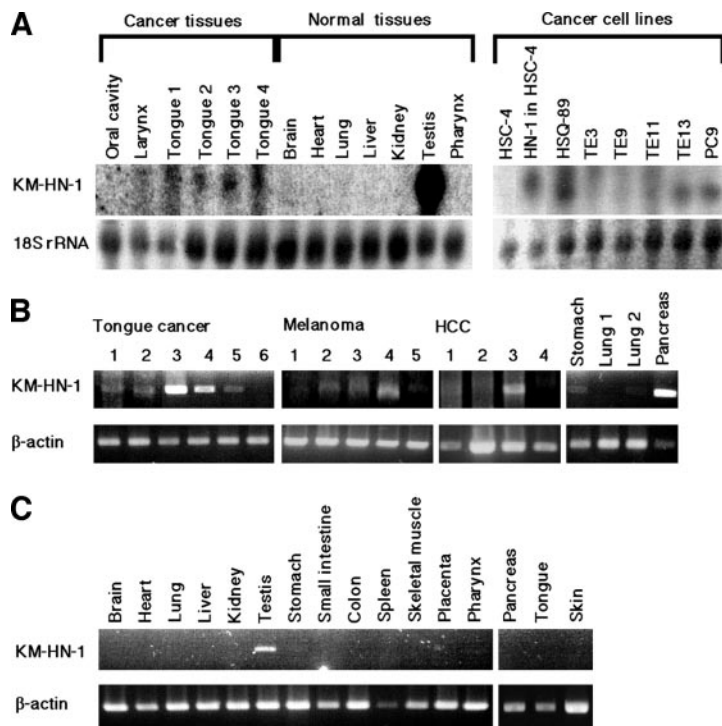
Fig. 2 Expression of *KM-HN-1* mRNA in normal and cancer tissues, and cancer cell lines. *A*, Northern blot analysis of *KM-HN-1* mRNA in various normal and cancer tissues, and cancer cell lines. The same filters were stripped and rehybridized with 18S rRNA to prove RNA integrity and to assess loading 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

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[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 $= (E\text{KM-HN-1})^{\Delta^{\text{CP}}\text{KM-HN-1}^{(\text{testis-sample})}} / (E\beta\text{-actin})^{\Delta^{\text{CP}}\beta\text{-actin}^{(\text{testis-sample})}} \times 100$.

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

Tissue type	KM-HN-1 expression (RT-PCR)* No. positive/no. tested		Anti-KM-HN-1 antibody†					
			Age mean	Absorbance ratio†		No. positive/ no. tested‡	*P* value	
	Tumor tissues	Cell lines		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.
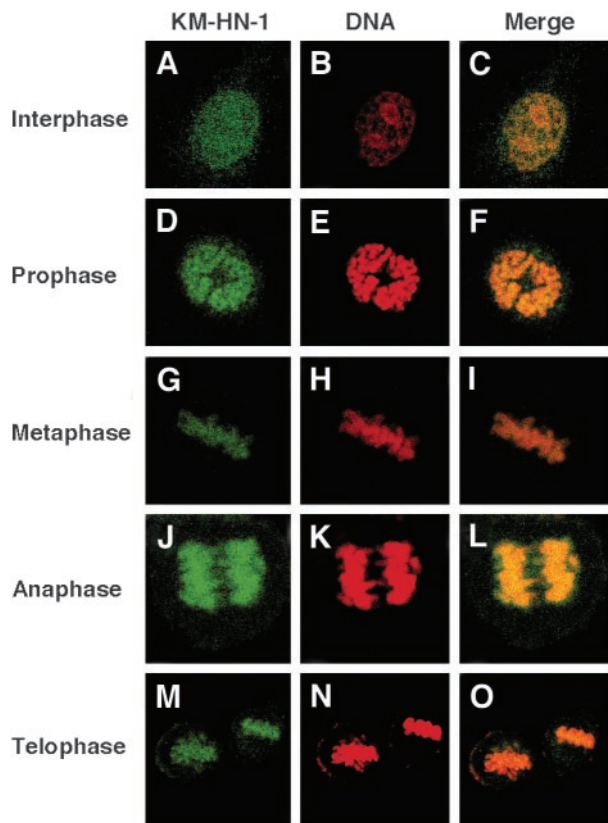
Fig. 3 Immunocytochemical analysis of KM-HN-1 protein with an esophageal cancer cell line, TE13, during mitosis: interphase (*A–C*), prophase (*D–F*), metaphase (*G–I*), anaphase (*J–L*), and telophase (*M–O*). TE13 cells were stained with anti-KM-HN-1 antibody (*green*). Chromosomes were visualized 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
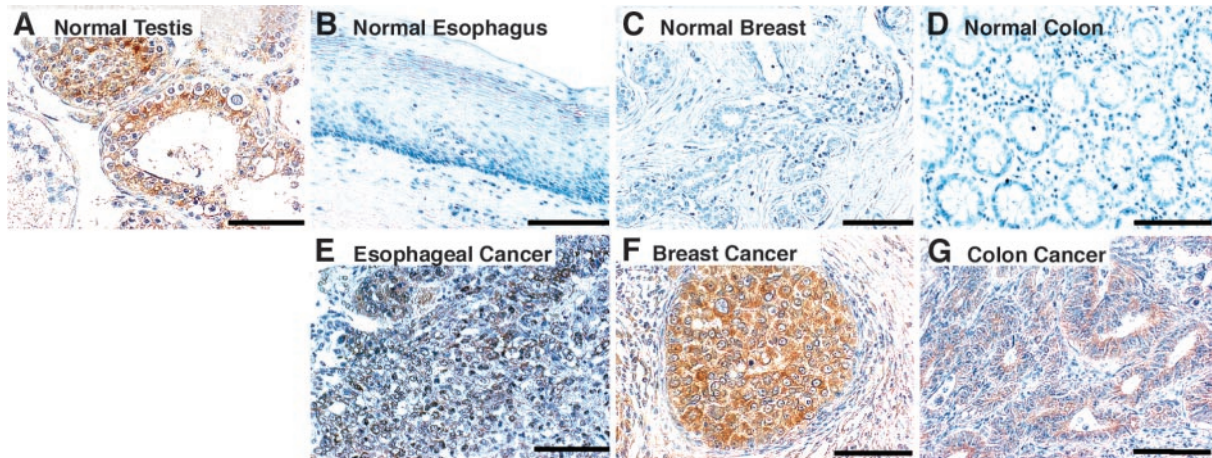
Fig. 4 Immunohistochemical staining of KM-HN-1 antigen in normal and cancer tissues by using avidin-biotin complex method and 3,3′-diaminobenzidine chromogen. *A.* in testis, seminiferous tubules were positively stained with strong intratubular staining of mostly spermatogenic cells, whereas no reactivity 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 immunoprotea-some 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
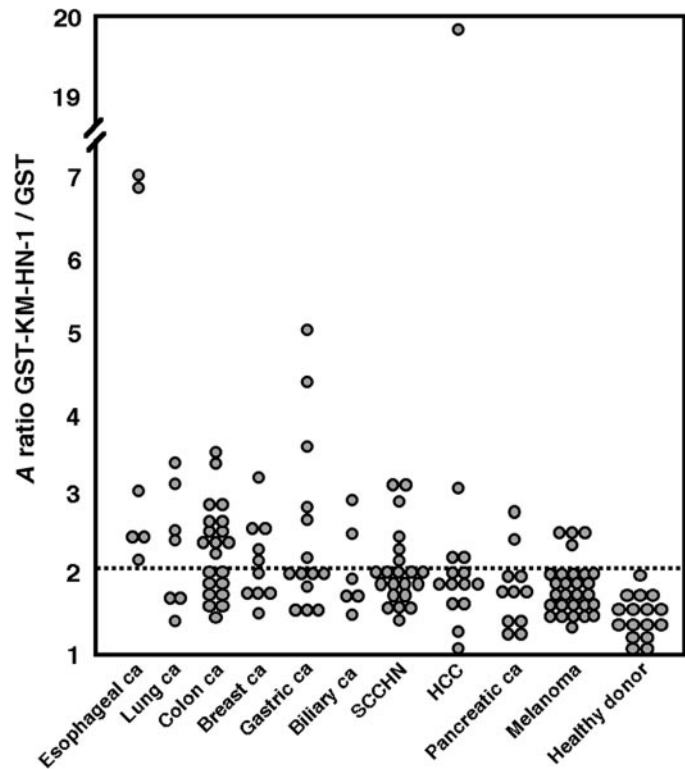
Fig. 5 Quantitation of KM-HN-1– specific IgG measured by ELISA in sera from age-matched healthy donors and patients with various types of cancer. Serum samples were diluted at 1:100 and were analyzed by ELISA to detect antibodies reactive to GST-KM-HN-1 fusion protein and GST alone. The ratio of the 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 = $(E_{KM-HN-1})^{\Delta CP_{KM-HN-1}^{(testis-sample)}} / (E_{\beta-actin})^{\Delta CP_{\beta-actin}^{(testis-sample)}} \times 100$.

† 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. 6C). 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. 8H 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
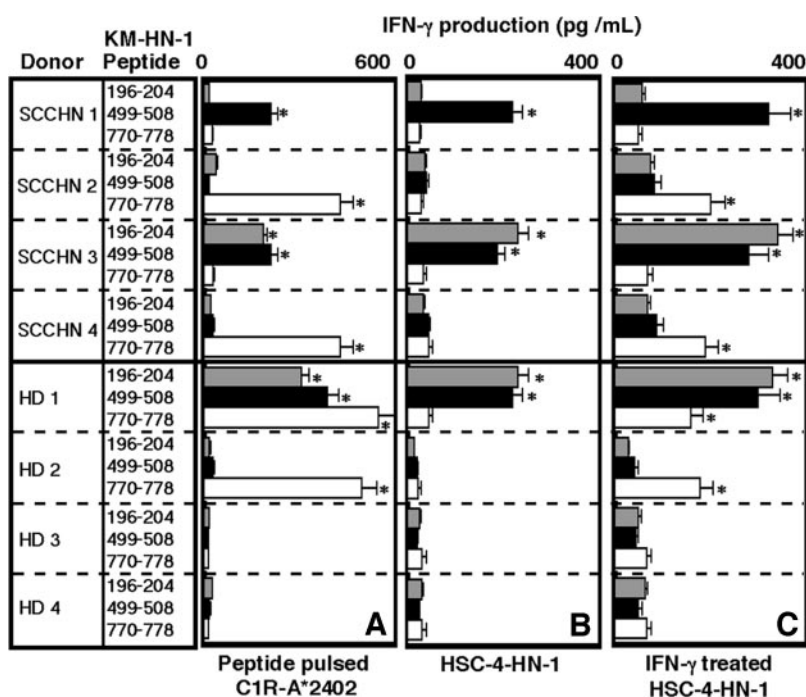
Fig. 6 Induction of CTLs by the KM-HN-1—derived peptides. CTL lines specific to three peptides, HN-1$_{196-204}$, HN-1$_{499-508}$, and HN-1$_{770-778}$, could be generated from HLA-A24–positive healthy donors and cancer patients. CTL responses to the peptide-loaded C1R-A*2402 cells (*A*), HSC-4 cells 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. *, $P < 0.05$.
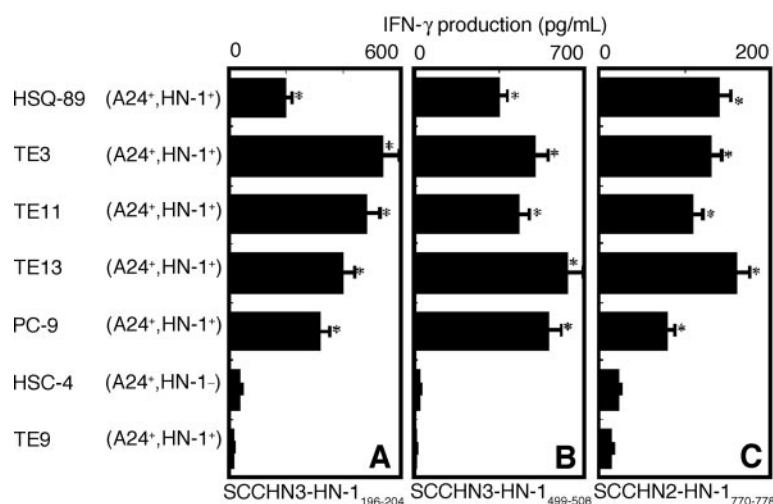
SCP-1 (sterol carrier protein-2). CENP-F protein localizes to the spindle midzone and the intracellular bridge in late anaphase and telophase, respectively. Localization of this protein suggests that it may play a role in chromosome segregation during mitosis (34, 35). Furthermore, autoantibodies against 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
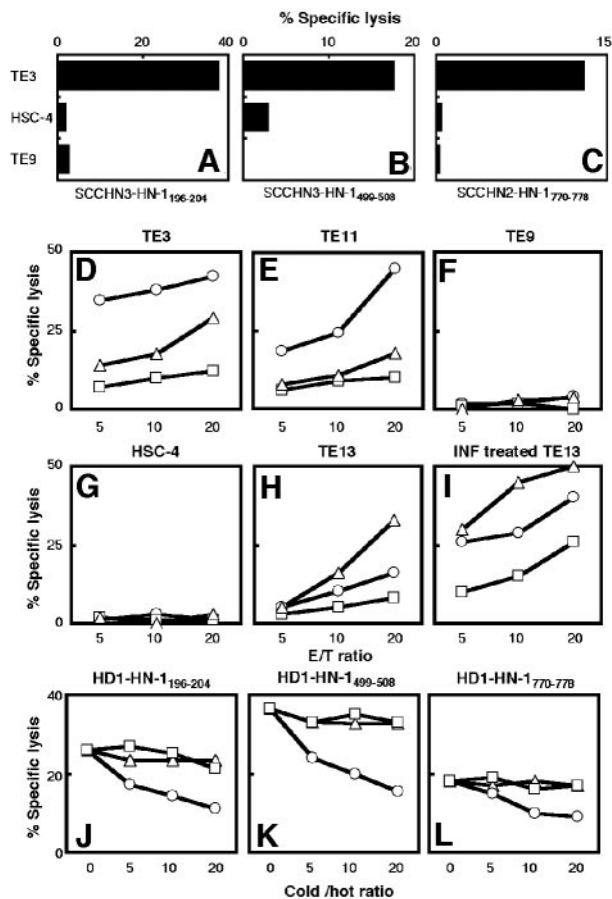
Fig. 8 ^{51}Cr release assay for cytotoxicity of CTL lines against cancer cell lines. *A–C*, cytotoxic activities of CTL lines generated from SC-CHN patients against cancer cell line were detected by 4-hour ^{51}Cr release assay at effector-to-target ratio of 10:1. Peptides used for CTL generation were 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.

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