Ran, a Small GTPase Gene, Encodes Cytotoxic T Lymphocyte (CTL) Epitopes Capable of Inducing HLA-A33–restricted and Tumor-Reactive CTLs in Cancer Patients

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ABSTRACT

Purpose: **The purpose is to identify a gene coding for tumor-associated antigen and peptide capable of inducing CTLs reactive to tumor cells with a HLA-A33–restricted fashion to provide scientific basis for specific immunotherapy to HLA-A33$^+$ cancer patients.**

Experimental Design: **An expression gene-cloning method was used to identify the tumor-associated antigen gene. Northern blot analysis and immunohistochemistry were used to examine the mRNA and protein expression levels in various cells and tissues, respectively. Synthetic peptides were examined for their ability to induce HLA-A33$^+$ tumor-reactive CTLs in peripheral blood mononuclear cells from cancer patients.**

Result: **A gene of small GTPase, *Ran*, which controls the cell cycle through the regulation of nucleocytoplasmic transport, mitotic spindle organization, and nuclear envelope formation, was found to encode epitopes recognized by the HLA-A33–restricted CTLs established from T cells infiltrating into gastric adenocarcinoma. The expression of the *Ran* gene was increased in most cancer cell lines and cancer tissues at both the mRNA and protein levels. However, it was not enhanced in the surrounding normal cells or tissues. It was also undetectable in normal tissues as far as tested. Ran-derived peptides at positions 48–56 and 87–95 could induce CD8$^+$ peptide-specific CTLs reactive to tumor cells from HLA-A33$^+$ epithelial cancer patients in a HLA class I-restricted manner.**

Conclusions: **Because of its increased expression in cancer cells and involvement in malignant transformation and/or the enhanced proliferation of cancer cells, the two Ran-directed peptides could be potent candidates in use for specific immunotherapy against HLA-A33$^+$ epithelial cancers.**

INTRODUCTION

There is growing evidence that human tumors express antigenic peptides recognized by CTLs, and some of these peptides have been used as peptide vaccines for cancer patients with the *HLA-A2* or *HLA-A24* allele (1–7). In contrast to the many reports on epitope peptides recognized by HLA-A2 or HLA-A24 CTLs (8–10), information on the antigens and peptides recognized by HLA-A33–restricted CTL is very limited (11–13). This lack of information has been hampering the development of peptide-based specific immunotherapy for HLA-A33$^+$ cancer patients, despite the relatively wide expression of the *HLA-A33* allele in people of various ethnicities (14% of Asians, 4% of Caucasians, and 16% of blacks; refs. 14, 15). To identify CTL epitope peptides in HLA-A33$^+$ cancer patients, we established a HLA-A33–restricted CTL line from the tumor-infiltrating lymphocytes (TILs) of a patient with scirrhous-type gastric adenocarcinoma, and identified a gene, *Ran* (16, 17), as a gene that codes for tumor-associated antigen (13). We have demonstrated in this study that the two Ran-derived peptides are capable of inducing HLA-A33–restricted CTL activity reactive to tumor cells in epithelial cancer patients.

MATERIALS AND METHODS

CTL Line and Tumor Cell Lines. The parental HLA-A33–restricted and tumor-reactive 850B-CTL line was established from the TILs of patient with scirrhous-type gastric adenocarcinoma (HLA-A*2402/A*3303, B7/B44, and Cw7/Cw14) by incubating TILs with interleukin 2 alone for >50 days by methods reported previously (13). Briefly, the patient's TILs were incubated with a culture medium (45% RPMI 1640, 45% AIM-V medium; Invitrogen, San Diego, CA; 10% FCS with 100 units/mL interleukin 2). These interleukin 2-activated TILs were tested for CTL activity every 7 days to various tumor cells by the standard 6-hour ^{51}Cr release assay. The cancer cell lines used for this experiment were as follows: gastric adenocarcinoma (MKN-28, MKN-45, SSTW-9, KATO-III, KWS, and

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HGC-27); lung adenocarcinoma (LC-1); lung squamous cell carcinoma (QG-56); head and neck carcinoma (KUMA-1); colon adenocarcinoma (SW620 and COLO 201); pancreatic adenocarcinoma (Panc-1); and human chronic myelogenous leukemia (K562). The HLA class I genotypes of these tumor cells have been described elsewhere (13). The expression of the HLA class I or HLA-A33 antigens on these cells was measured by flow cytometry on a FACScan (Becton Dickinson, San Jose, CA) after the cells were stained with anti-HLA class I (W6/32) monoclonal antibody (mAb), recognizing a monomorphic region of the HLA class I molecule, or anti-HLA-A33 mAb (IgM; One Lambda, Canoga Park, CA), recognizing a polymorphic region of the HLA-A33 molecule, as reported previously (13). The responses of the CTL line to various cancer and normal cells were tested by using both a 6-hour ^{51}Cr release assay and an IFN-γ measurement with an ELISA (limit of sensitivity: 10 pg/mL), as reported previously (13).

Identification of the *Ran* Gene. An expression gene-cloning method was used to identify the gene coding for the tumor antigen recognized by the HLA-A33–restricted and tumor-specific 850B-CTL subline, 850B-CTL1, as reported previously (13). In brief, poly $(A)^+$RNA of LC-1 lung adenocarcinoma cells was converted to cDNA, ligated with the *Sal*I adapter, and inserted into the expression vector pSV-SPORT-6 (Invitrogen). cDNA of *HLA-A*3303* or *HLA-A*2601* was obtained by reverse transcription-PCR with RNA from KUMA-1 or KE-4 cells, respectively, and then cloned into the eukaryotic expression vector pCR3.1 (Invitrogen). Two hundred nanograms of plasmid DNA clones of the LC-1 cDNA library and 200 ng of the *HLA-A*3303* or *HLA-A*2601* cDNA were mixed with 1 μL of Lipofectamine (Invitrogen) in 120 μL of Opti-MEM (Invitrogen) for 40 minutes. The COS-7 cells (5×10^3) were incubated with 50 μL of the mixture for 6 hours, followed by the addition of 150 μL of RPMI 1640 containing 10% FCS. A total of 1×10^5 clones from the LC-1 cDNA library was tested for their ability to stimulate IFN-γ production by 850B-CTL1 after cotransfection with the *HLA-A*3303* cDNA into the COS-7 cells in the first screening, and significant levels of IFN-γ production were obtained in 10 different wells. At the second screening, each of the cDNA pools from each of the positive wells was subdivided into 100 different wells in 96 flat-bottomed plates (expected number of clones per well: one clone per well) in duplicate and tested for their ability to stimulate IFN-γ production. After the second screening, two positive clones were identified for additional analyses. The both strands of DNA sequencing were performed by a dideoxynucleotide sequencing method using a DNA sequence kit and ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Foster, CA). This article describes the results for one of the cloned genes, the *Ran* gene.

Northern Blot Analysis and Immunohistochemistry. The mRNA expression of *Ran* on various tumor or normal tissues (Multiple Tissue Northern Blots; Clontech, Tokyo, Japan) was examined by Northern blot analysis with a ^{32}P-labeled *Ran* probe by the method as reported previously (9). The expression of Ran protein in various cancer or normal tissues was evaluated by immunohistochemistry on 10% formalin-fixed, paraffin-embedded tissue sections. After the tissue sections were pretreated with heat for antigen unmasking, immunohistochemistry was performed using the Ventana automated immunostainer (Ventana Medical Systems, Tucson, AZ) with anti-Ran antibody (1:160 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and second antibody (Antigoat Omunitag Plus; Shandon, Pittsburgh, PA).

Determination of the Antigenic Peptides. Among the possible peptide sequences with motifs for binding to the HLA-A33 molecule (18, 19) in the deduced amino acid sequence of Ran, seven different peptides that showed stronger binding activity for HLA-A33 in a computer analysis (Bioinformatics and Molecular Analysis Section; NIH, Bethesda, MD) were used. Peptides with a purity of >95% were obtained from Biologica (Nagoya, Japan). For the peptide-binding assay, RMA-S-A33 cells (1×10^4 cells per well) and RMA-S TAP (transporter-associated peptides processing)-deficient mouse lymphoma cells stably transfected with *HLA-A*3303* cDNA were used as reported previously (13). Briefly, the cells were incubated at 26°C for 18 hours. After being washed with PBS, the cells (1×10^6 cells) were suspended in Opti-MEM containing 3 μg/mL human β_2-microglobulin and 10 μg/mL peptides, followed by incubation at 26°C for 3 hours, and at 37°C for 3 hours. After being washed with PBS, the cells were then incubated with anti-HLA-A33 mAb at 4°C for 30 minutes, then with FITC-conjugated rabbit antimouse IgM antibody (Cappel, Aurora, OH) at 4°C for 30 minutes. The cells were analyzed by FACScan, and their binding activity was evaluated by the mean fluorescence intensity. The cells cultured only at 26°C were used as a positive control. For the detection of antigenic peptides recognized by the 850B-CTL1, C1R-A3303 cells (1×10^4 cells per well), C1R human multiple myeloma cells stably transfected with *HLA-A*3303* cDNA (13), were cultured with the indicated concentrations of peptides. Two hours later, the 850B-CTL1 cells (2×10^5 cells per well) were added and incubated for an additional 18 hours. Levels of IFN-γ in the culture supernatants were measured by ELISA.

Induction of CTL by Peptides. After written informed consent was obtained, peripheral blood mononuclear cells (PBMCs) from 10 HLA-$A33^+$ cancer patients were obtained and used for the CTL induction assay by a new method for detection of peptide-specific CTL precursor cells as reported previously (20), which allowed to detect peptide-specific CTL precursor cells without addition of antigen-presenting cells in culture and thus could save the numbers of PBMCs needed for the assay. In brief, PBMCs (1×10^5cells per well) were stimulated with each of the indicated Ran peptides. On the 14th day of the culture, the cells from each well were washed, independently collected, and divided into the four equal portions. Two such portions were separately tested for their ability to produce IFN-γ in response to C1R-A33 pulsed with a corresponding peptide, whereas the remaining two portions were tested with a negative control peptide (RAN21-29). After an 18-hour incubation, the supernatant was collected for the measurement of IFN-γ by ELISA. The well of successful induction of peptide-specific CTL was judged to be positive when the mean value of supernatant of well showed >100 pg/mL IFN-γ production with P of < at least 0.05. The PBMCs showing a positive response were collected and additionally cultured with interleukin 2 alone for 10 to 14 days for a standard 6-hour ^{51}Cr release assay (13). For the inhibition test, 20 μg/mL anti-HLA class I (W6/32,

IgG2a), anti-HLA-A2 (BB7.2, IgG2b), anti-CD8 (Nu-Ts/c, IgG2a), anti-HLA class II (H-DR-1, IgG2a), and anti-CD4 (Nu-Th/i, IgG1) mAbs were used. The anti-CD14 (JML-H14, IgG2a) mAb served as a control. A two-tailed Student's *t* test was used for the statistical analysis.

RESULTS

Identification of a Gene Recognized by 850B-CTL1. A HLA-A33–restricted and tumor-specific CTL line was established from the TIL of a patient (HLA-A2402/A3303, B7/B44, and Cw7/Cw14) with scirrhous-type gastric adenocarcinoma as reported previously (13). One of the sublines (850B-CTL1) with the $CD3^+CD4^-CD8^+$ phenotype showed HLA-A33–restricted and tumor-specific CTL activity as measured by ^{51}Cr release assay. As shown in Fig. 1*A*, the 850B-CTL1 showed cytotoxicity against LC-1 and KUMA-1 cells but not against any of the $HLA\text{-}A33^-$ tumor cells, COS-7 cells, natural killer target cell line K562, or $HLA\text{-}A33^+$ phytohemagglutinin-activated T cells (phytohemagglutinin blast cells) from the PBMCs of healthy donors. A total of 1×10^5 cDNA clones from the cDNA library of LC-1 tumor cells was tested for their ability to stimulate IFN-γ production by this 850B-CTL1 when cotransfected with *HLA-A*3303* into COS-7 cells. After repeated experiments for several candidate clones, one clone (*clone 1*) was confirmed to encode an antigen recognized by the HLA-A33–restricted 850B-CTL1. As shown in Fig. 1*B*, COS-7 cells transfected with *clone 1* and *HLA-A*3303*, but not with *clone1* and *HLA-A*2601* as a negative control, induced IFN-γ production in 850B-CTL1 in a dose-dependent manner. In contrast, COS-7 cells transfected with either *clone 1* or *HLA-A*3303* alone were not recognized by 850B-CTL1 (Fig. 1*B*). In addition, the other clones from the LC-1 cDNA library used as a negative control failed to induce IFN-γ production in 850B-CTL1 when cotransfected with *HLA-A*3303* into COS-7 cells. The result from this negative *clone 2* is shown as one representative clone in Fig. 1*B*. The nucleotide sequencing of *clone 1* revealed 1035-bp full length of cDNA by searching the GenBank (GenBank accession no. BC014901), which is completely identical to that of *Ran*, a small GTPase that regulates nucleocytoplasmic transport, mitotic spindle organization, and nuclear envelope formation (16).

Overexpression of Ran Protein in Tumor Tissues. The mRNA expression of the *Ran* gene in normal and cancer cells was investigated by Northern blot analysis. As shown in Fig. 2*A*, a band of ~1 kb was clearly detected in all of the normal tissues tested, except the peripheral blood lymphocytes (Fig. 2*A*, *Lane 12*), with specially higher expression in the skeletal muscle (Fig. 2*A*, *Lane 3*), kidney (Fig. 2*A*, *Lane 7*), liver (Fig. 2*A*, *Lane 8*), and placenta (Fig. 2*A*, *Lane 10*) and lower expression in the colon (Fig. 2*A*, *Lane 4*), small intestine (Fig. 2*A*, *Lane 9*), and lung (Fig. 2*A*, *Lane 11*). In addition, this gene was highly expressed in most of the adenocarcinoma and squamous cell carcinoma cell lines tested from various organs, including the stomach (Fig. 2*B*, *Lanes 2–5*, *12*), lung (Fig. 2*B*, *Lanes 6* and 7), head and neck (Fig. 2*B*, *Lane 8*), pancreas (Fig. 2*B*, *Lane 9*), and

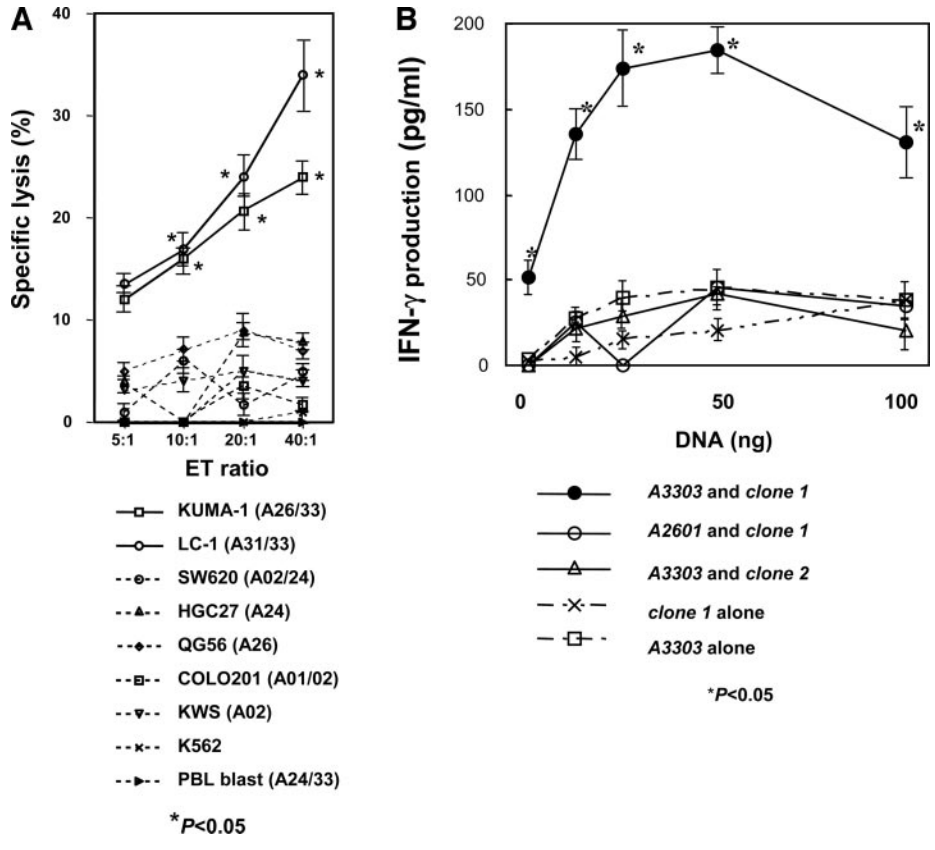

Fig. 1 Identification of the gene recognized by the HLA-A33–restricted 850B-CTL1. *A*. The cytotoxic activity of 850B-CTL1 against various target cells was tested by a 6-hour ^{51}Cr release assay at different E:T ratios. Values represent the means of triplicate determinations. Statistical analysis was performed by a two-tailed Student's *t* test. $*P < 0.05$. Three independent experiments were carried out with the consistent results, and one of the representative results was given in this figure. *B*. COS-7 cells that had been cotransfected with the indicated amounts of *clone 1* (or *clone 2*) and 100 ng of *HLA-A*3303* (or *HLA-A*2601* as a negative control) were tested for their ability to stimulate IFN-γ production by 850B-CTL1. Values represent the means of triplicate determinations. Statistical analysis was performed by a two-tailed Student's *t* test. $*P < 0.05$. Two independent experiments were carried out with the consistent results, and one of the representative results was given in this figure.

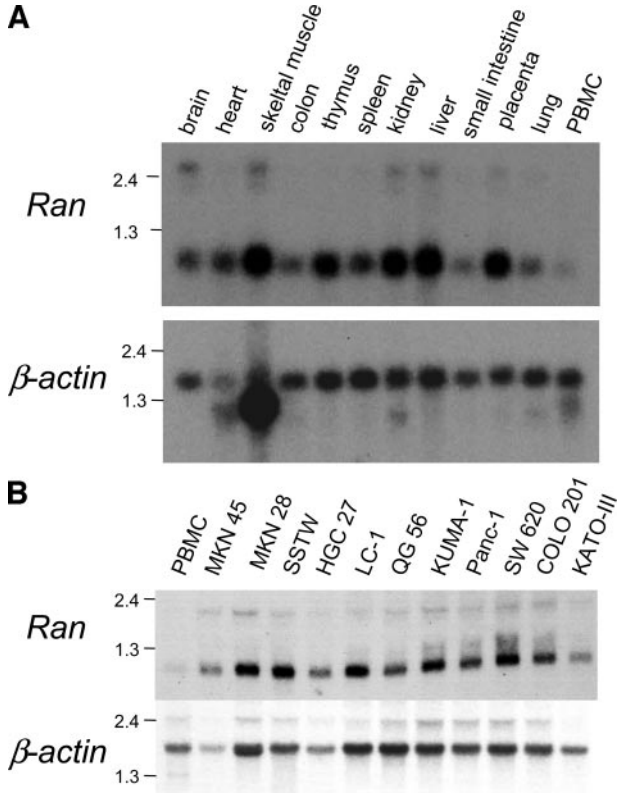

Fig. 2 Expression of *Ran* mRNA. *A.* The expression of *Ran* or control β-*actin* mRNA in normal tissues was examined by Northern blot analysis. *B.* The expression of *Ran* or control β-*actin* mRNA in a panel of cancer cell lines was examined by Northern blot analysis.

colon (Fig. 2*B*, *Lanes 10* and *11*). These results indicate that this gene is expressed in the majority of cancer cells and is also expressed in most normal tissues at various levels. The expression of the *Ran* gene at the protein level in both cancer (Fig. 3*A–E*) and normal tissues (Fig. 3*F–I*) from various organs were investigated by immunohistochemistry. The Ran protein was highly and selectively expressed in various types of cancer cells in tumor tissues, including gastric (Fig. 3*A*), colon (Fig. 3*B*), pancreas (Fig. 3*C*), and lung cancer tissues (Fig. 3, *D* and *E*) but not in the normal cells in these tumor tissues. In contrast, it was not detectable in any normal tissues tested (Fig. 3*F*, gastric; Fig. 3*G*, colon; Fig. 3*H*, pancreas, and; Fig. 3*I*, lung).

Identification of Ran-derived Antigenic Peptides Recognized by the 850B-CTL1. To identify the Ran-derived CTL epitopes, we determined seven possible peptide sequences with motifs for binding to the HLA-A33 molecule in the deduced amino acid sequence of Ran in a computer analysis. Each of the 7 different Ran-derived synthetic peptides with binding motifs to the HLA-A33 molecule was loaded onto HLA-A33-transfected cells, RMA-S-A33, at a concentration of 10 μmol/L, and the binding affinities of these peptides were analyzed. As shown in Table 1, all of the 7 peptides were able to bind to the RMA-S-A33 cells with slightly different affinities. Next, *HLA-A*3303*–transfected C1R cells were incubated with each of these peptides at a concentration of 1 μmol/L, and their ability to induce IFN-γ production by 850B-CTL1 was tested. Four of these peptides, Ran48-56, Ran87-95, Ran97-106, and Ran121-129, induced significant levels of IFN-γ production (Fig. 4*A*) in a dose-dependent manner (Fig. 4*B*). The optimal concentration of Ran48-56, Ran87-95, and Ran97-106 peptides for loading onto the *HLA-A33*–transfected C1R cells was 1 μmol/L, whereas that of Ran121-129 peptide, which has the lowest binding affinity among the tested 8 peptides (Table 1), was higher than 1 μmol/L (>10 μmol/L) under the used condition. Additional experiments including the use of CTL lines or clones specific to each peptide and also different doses of peptides are needed to confirm the optimal concentration of each peptide. None of the remaining three peptides stimulated 850B-CTL1 enough to produce significant levels of IFN-γ (data not shown).

Induction of CTL by Ran-derived Peptides. The Ran48-56, Ran87-95, Ran97-106, and Ran121-129 peptides were then tested for their ability to induce HLA-A33–restricted and tumor-specific CTLs in the PBMCs of 10 HLA-A33$^+$ epithelial cancer patients. Ran48-56 and Ran87-95 stimulated PBMCs from 4 and 5 of 10 cancer patients tested to produce significant amounts of IFN-γ by recognition of the *HLA-A33*–transfected C1R cells loaded with the corresponding peptides, respectively (Table 2). In contrast, the other two peptides induced peptide-reactive CTLs from only 1 of 10 patients tested. The experiments were carried out twice, and one of the representative results is given in Table 2

Next, the CTL activity against tumor cells in the patients' PBMCs stimulated with Ran-derived peptides was examined by a 6-hour ^{51}Cr release assay. The PBMCs stimulated with either the Ran48-56 or Ran87-95 peptide showed significant levels of cytotoxicity against the HLA-A33$^+$ LC-1 tumor cells but not against either HLA-A33$^-$ QG56 cells or the HLA-A33$^+$ phytohemagglutinin blast cells in all of the cases in which CTL activities were observed by IFN-γ production assay. Representative results of the three cases are given in Fig. 5. In contrast, PBMCs stimulated with a negative control peptide (Ran21-29) did not show any specific CTL activity (Fig. 5).

Furthermore, the restriction and peptide specificity of the cytotoxicity were confirmed by inhibition and competition assays, respectively. The levels of cytotoxicity mediated by the PBMCs stimulated with both Ran48-56 and Ran87-95 were significantly inhibited by anti-HLA class I (W6/32) or anti-CD8 mAb but not by the other mAb tested. Furthermore, the cytotoxicity was inhibited by the addition of the corresponding peptide-pulsed C1R-A3303 cells but not by that of the Ran21-29 peptide-pulsed cells in all of the cases tested. Representative results on patient 4 are given in Fig. 6. These results suggest that the cytotoxicity induced by these peptides was largely mediated by the peptide-reactive CD8$^+$ T cells in an HLA class I-restricted manner.

DISCUSSION

The present study demonstrated that *Ran* encodes tumor antigenic epitopes recognized by HLA-A33–restricted and tumor-specific CTL established from T cells infiltrating into gastric adenocarcinoma. *Ran* is a small GTPase, which has been known to be one of the most highly conserved proteins in eukaryotes and to be essential for cell viability in all organisms

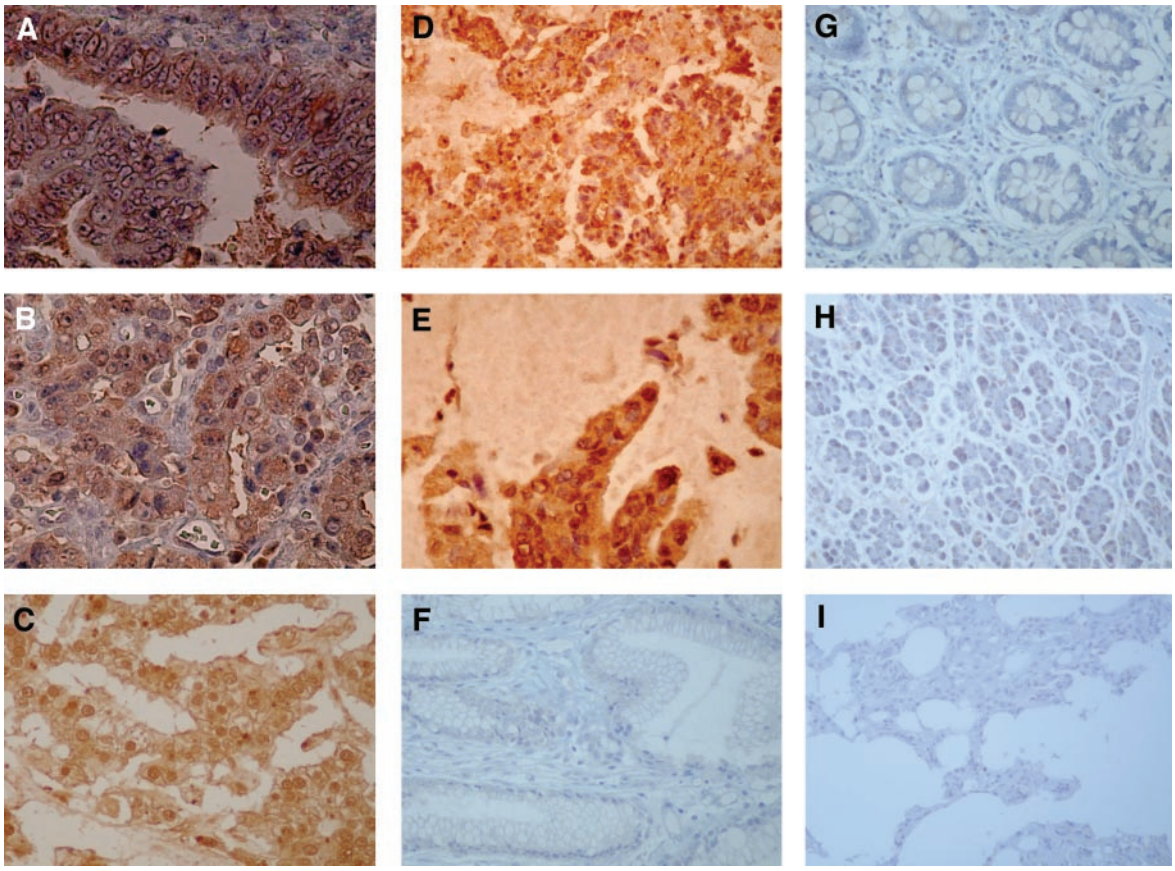

Fig. 3 Expression of Ran protein. Expression of Ran protein was examined by immunohistochemistry with anti-Ran mAb. Representative results of the histochemical stainings in cancer tissues (*A*, gastric; *B*, colon; *C*, pancreas; *D*, lung adenocarcinoma; *E*, lung squamous cell carcinoma) and normal tissues (*F*, gastric; *G*, colon; *H*, pancreas; *I*, lung) are shown.

tested (21). Previous reports have shown that *Ran* is involved in various cellular processes, including nucleocytoplasmic transport, mitotic spindle organization, and nuclear envelope formation (16). In view of its crucial role in cell cycle progression, *Ran* may contribute to malignant transformation and/or enhanced proliferation in cancer cells. For example, a direct association between *Ran* and viral oncoproteins such as human adenovirus E1A, human papillomavirus E7, and SV40 large T antigen has been recently reported to be closely associated with cellular transformation and genomic instability induced by viral infection (22).

Northern blot analysis revealed that the strong band of the 1.2-kb size, which was the expected size of *Ran* mRNA, was ubiquitously detected in both tumor cell lines and normal tissues and cells with a low expression of PBMCs. In addition, two bands at the sizes of 1.9 and 2.4 kb were dimly detectable in both normal and cancer tissues. The smaller 1.9-kb size band was compatible with that of 18S rRNA. The 2.4-kb band was also observed, but the longest *Ran* mRNA reported in the literature was 1.65 kb (NM006325), and the theoretically maximum length of *Ran* mRNA is ~2.0 kb. These results suggest that these other bands are nonspecific. In the expression analyses, we showed that *Ran* is highly expressed in most of the cancer cell lines or cancer tissues tested at both the mRNA and protein levels. In contrast, regardless of its expression in normal tissues at the mRNA level, it was not enhanced in the surrounding normal cells or tissues. It was also undetectable in normal tissues as far as

Table 1 Binding activity of the Ran-derived peptides to the RMA-S-A33 cells

Peptide	Mean fluorescence intensity
(−), 26°C	16.4
(−), 37°C	4.2
TRP2 197-205 (LLGPGRPYR)	15.6
Ran 21-29 (TGKTTFVKR)	15.6
Ran 48-56 (HPLVFHTNR)	14.6
Ran 68-76 (GQEKFGGLR)	14.8
Ran 87-95 (IIMFDVTSR)	14.5
Ran 97-106 (TYKNVPNWHR)	15.0
Ran 101-110 (VPNWHRDLVR)	16.1
Ran 121-129 (GNKVDIKDR)	14.0

NOTE. Binding activity of the Ran-derived peptides to the HLA-A33 molecules was evaluated by the mean fluorescence intensity after staining of the RMA-S-A33 cells pulsed with the indicated peptide with anti-HLA-A33 mAb. The cells cultured only at 26°C were used as a positive control. The cells cultured at 26°C for 3 hours followed by 37°C for 3 hours without peptide were used as a negative control.

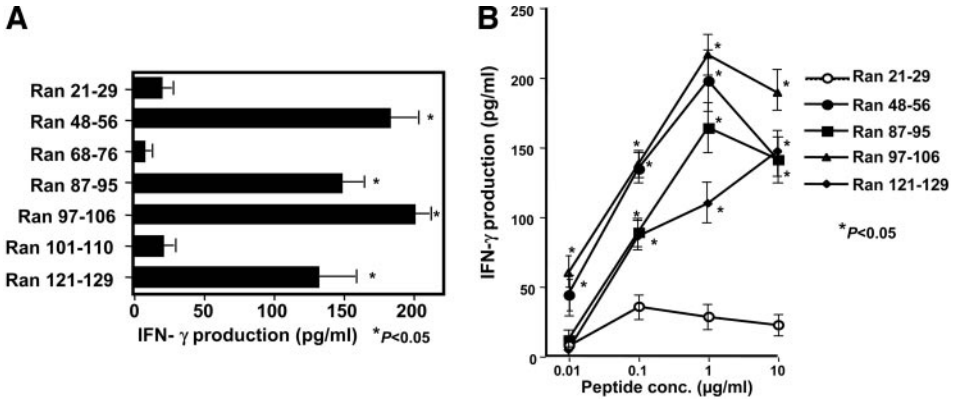

Fig. 4 Identification of CTL epitopes. *A*, determination of the antigenic peptides. Each of the seven peptides derived from Ran was loaded onto C1R-A33 cells at a concentration of 1 μg/mL. The 850B-CTL1 cells were cultured with the peptide-loaded C1R-A33 for 18 hours, and the culture supernatant was harvested to measure IFN-γ using an ELISA. The background of the IFN-γ production (<50 pg/mL) by 850B-CTL1 in response to the peptide-unloaded C1R-A33 cells was subtracted from the values. Values represent the means of triplicate assays. *B*, dose-dependent production of IFN-γ by the 850B-CTL1 in response to each of the four Ran peptides. The indicated doses of the Ran-derived peptides, Ran48-56, Ran87-95, Ran97-106 and Ran121-129, and Ran 21-29 (control) were loaded onto C1R-A33 cells, and the ability of the peptides to stimulate IFN-γ production by the 850B-CTL1 cells was tested. The background of the IFN-γ production (<50 pg/mL) by 850B-CTL1 in response to the peptide-unloaded C1R-A33 cells was subtracted from the values. Values represent the means of triplicate assays. Statistical analysis was performed by a two-tailed Student's *t* test. $*P < 0.05$. The experiments were performed twice at different times with the consistent results, and one of the representative results was given in this figure.

tested. This discrepancy in the expression pattern has also been observed in several other tumor-associated antigens such as SART1, SART3, and IEX-1, as reported previously (9, 10, 13). Similar to our findings, Li *et al.* (23) demonstrated that prostate cancer tissues showed significantly higher expression levels of Ran proteins than benign tissues. In addition, Ran binding proteins such as RanBP7 and RanBPM have been reported to be preferentially expressed in cancer tissues and to be associated with the increased proliferation of cancer cells (24, 25). In view of the finding that Ran and its associated proteins are strongly and selectively expressed in various cancer tissues, Ran may be one of the ideal target molecules for the treatment of patients with cancer.

Because *Ran* is also expressed in normal tissues at the mRNA level, particularly in the skeletal muscle, kidney, and liver, these organs may be possible targets for the adverse effects of specific immunotherapy with Ran-derived antigenic epitopes. No severe adverse effects on normal tissues or organs have been observed in our clinical trials with peptide vaccines derived from tumor-associated antigens, some of which are expressed in normal tissues and organs (4–7). Those antigens included the cyclophilin B (26), Lck (27), and prostate acid phosphate (28). Some of the other antigens were preferentially expressed in tumor cells but not in normal cells. Those antigens included SART-2 (29), SART-3 (10), and MRP-3 (30). Ran belonged to the latter group. However, no induction of autoimmunity in the clinical trials for HLA-A24 and HLA-A2 cancer patients past studies does not guarantee the safety of Ran-derived peptides reported in this study. As far as reports searched at the literature levels, vaccination of self-antigen–derived peptides for HLA-A33 cancer patients is not reported. Therefore, careful clinical trials shall be conducted to determine the safety of Ran-derived peptides for cancer patients.

Among the seven peptides with HLA-A33 binding motifs tested, only four peptides, Ran48-56, Ran87-95, Ran97-106, and Ran121-129, were recognized by 850B-CTL1. All of the seven peptides tested have similar binding activities to HLA-A33 molecules, and thus, this result cannot merely be explained by the differences in binding affinities between the peptides and HLA-A33 molecules. Only the four peptides that were recog-

Table 2 Induction of peptide-reactive CTLs

	Patient no.										
Peptide	1 Lung	2 Cervical	3 Uterus	4 Prostate	5 Lung	6 Prostate	7 Prostate	8 Gastric	9 Prostate	10 Prostate	Total
RAN48-56	44	246	123	103	38	27	67	134	17	23	4/10
RAN87-95	51	29	4	157	15	159	118	95	114	16	5/10
RAN97-106	35	102	21	87	21	133	53	44	39	75	1/10
RAN121-129	161	71	17	54	68	18	23	39	37	53	1/10

The PBMCs from patients were tested for their reactivity to a corresponding peptide after *in vitro* stimulation with each peptide for 12 days. Values represent the IFN-γ production by the effector PBMCs in response to the C1R-A33 cells prepulsed with the corresponding peptide. The background IFN-γ response to the C1R-A33 cells prepulsed with RAN21-29 negative control peptide was subtracted (<50 pg/mL), and the results which showed the best response among four wells are shown. Significant values ($P < 0.05$ by two-tailed Student's *t* test) are underlined.

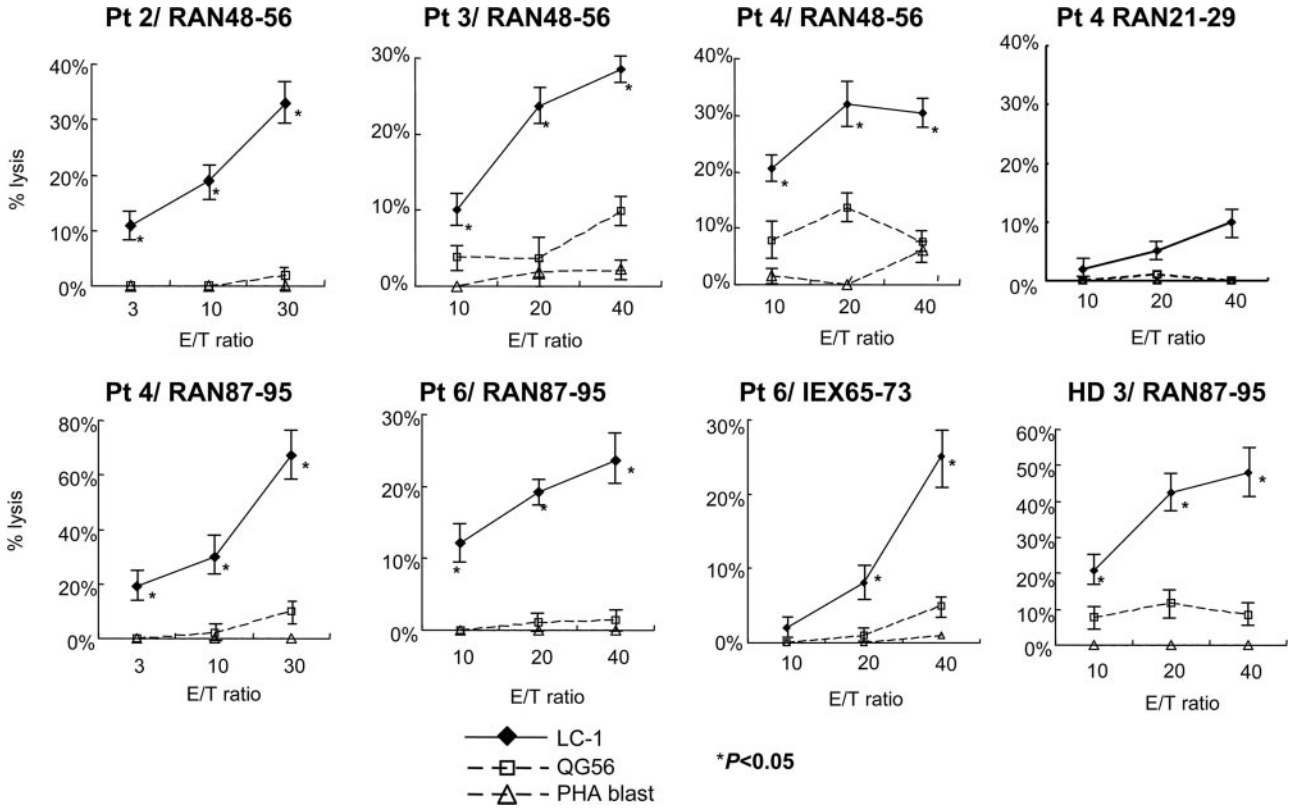

Fig. 5 Cytotoxity of the peptide-induced CTLs. Peptide-specific CTLs were induced by the Ran-derived peptides, Ran48-56 and Ran87-95, in the PBMCs from cancer patients. The cytotoxic activity of the peptide-induced CTLs against the LC-1 (HLA-A33$^+$) and QG56 (HLA-A33$^-$) tumor cells was measured by a 6-hour ^{51}Cr release assay at different E:T ratios. Values represent the means of triplicate determinations. Statistical analysis was performed by a two-tailed Student's *t* test. $*P < 0.05$. Two independent experiments were carried out with the consistent results, and one of the representative results was given in this figure.

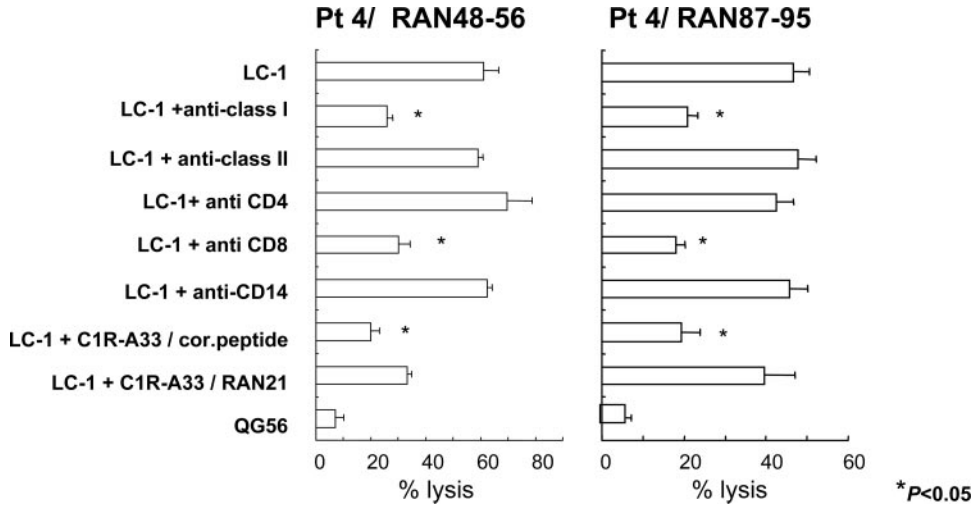

Fig. 6 Inhibition and competition assays. For the inhibition test, the peptide-stimulated PBMCs were tested for their restriction and for the peptide specificity of cytotoxicity against LC-1 (HLA-A33$^+$) by a standard 6-hour ^{51}Cr release assay in the presence or absence of 20 μg/mL anti-HLA class I (W6/32, IgG2a), anti-HLA class II (H-DR-1, IgG2a), anti-CD4 (Nu-Th/i, IgG1), and anti-CD8 (Nu-Ts/c, IgG2a) mAbs. Anti-CD14 (JML-H14, IgG2a) mAb served as a negative control. For the competition assay, unlabeled C1R-33 cells pulsed with the corresponding peptide or the RAN21-29 peptide as a negative control were added to the ^{51}Cr release assay at a cold-to-hot target cell ratio of 10 to 1. The 6-hour ^{51}Cr release assay was performed at an E:T ratio of 10 to 1. Values represent the means ± SD of percentage specific lysis of triplicate determinations. Statistical analysis was performed by a two-tailed Student's *t* test. $*P < 0.05$. Two independent experiments were carried out with the consistent results, and one of the representative results was given in this figure.

nized by 850B-CTL1 may be generated through the natural antigen-processing machinery *in vivo* and expressed in complex with the HLA-A33 molecules on the cell surfaces of antigen-presenting or tumor cells. However, additional studies with a relatively large numbers of PBMCs from different types of cancers are needed to confirm this issue and also for possible application of the remaining three peptides in use for peptide vaccination.

HLA-A33 is one of the most common HLA-A alleles in Asians and blacks and is found in 13% of Japanese, 14% of Koreans, 4% of Caucasians, and 16% of blacks (14, 15). The two Ran-derived peptides induced HLA-A33–restricted and tumor-specific CTLs in the PBMCs of epithelial cancer patients. Because the Ran protein is preferentially expressed in cancer tissues and is suggested to be associated with the malignant transformation and/or enhanced proliferation of cancer cells (16), these peptides might be appropriate target molecules to use for the peptide-based specific immunotherapy of HLA-A33$^+$ cancer patients.

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