

**Fig. 6** Cytotoxicity by the peptide-stimulated PBMCs. Each of these eight peptides was tested for its ability to induce CTL activity in PBMCs of five HLA-A2<sup>+</sup> colon cancer patients. Their HLA-A2 genotypes were determined by a previously reported method (10); patients 2 and 4 had the A0201 genotype; patients 3 and 5 had the A0206 genotype; and patient 1 had the A0207 genotype. PBMCs were stimulated with each of the eight peptides (10 μg/mL) and IL-2 (100 units/mL) every 3 days for 15 days, followed by incubation with IL-2 alone for an additional 15 days, and tested for their cytotoxicity against HLA-A2<sup>+</sup> SW620 tumor cells in the presence or absence of various mAbs by a standard 6-hour <sup>51</sup>Cr release assay at an effector to target cell ratio of 10:1 in triplicate assays. HLA-A2<sup>-</sup> QG56 tumor cells and HLA-A24<sup>+</sup> PHA-blast cells were used as negative controls for the <sup>51</sup>Cr release assay. The peptide specificity of the cytotoxicity was also confirmed by competition assay by addition of T2 cells pulsed with a corresponding peptide or a HIV peptide as a negative control at a hot to cold cell ratio of 20:1 in triplicate assays. Results for the AICRT/I peptides (panel A) and for the TYMS or PKG1 (panel B) peptides that had the ability to induce CTLs from at least two patients are shown in Fig. 6. A two-tailed Student's *t* test was used for the statistical analysis in this study; *P* values of <0.05 were considered to indicate statistical significance. Values represent the means ± SD of the percentage of specific lysis.

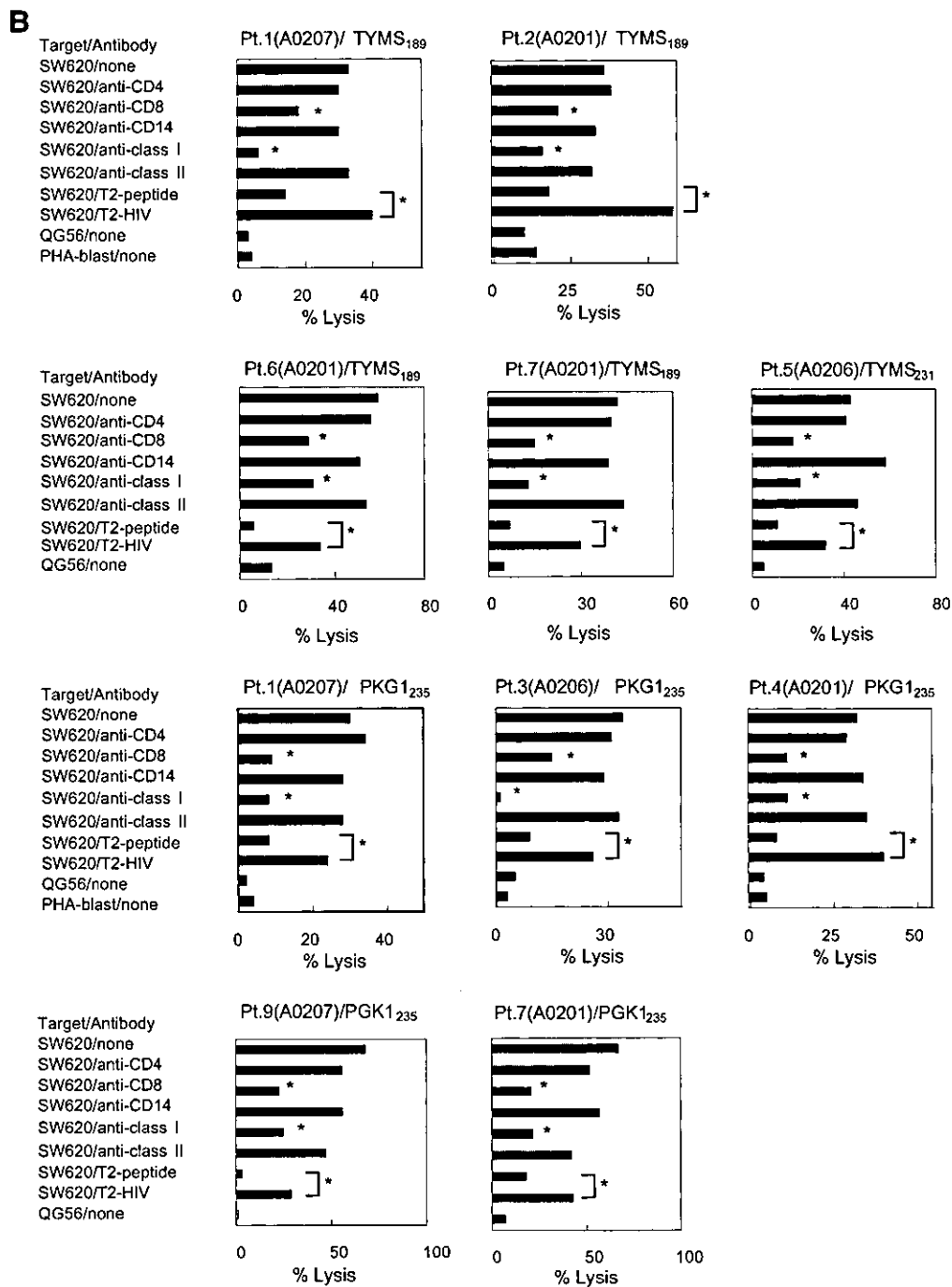


Fig. 6 Continued.

ously proliferate, differentiate, and mature and then quickly turnover via apoptosis. Therefore, adverse events such as diarrhea are expected to be associated with vaccination of peptides derived from proliferation-related proteins. However, no severe adverse effects were observed in our phase I clinical trials of individualized peptide vaccination for advanced cancer patients, which were performed using peptides from proliferation-related proteins (9, 21–25). However, whether or not proliferation-

related peptides are suitable as vaccines for the treatment of colon cancer needs to be carefully evaluated through additional basic and clinical studies. It also remains to be elucidated whether or not these CTL epitope peptides derived from proteins ubiquitously expressed in both tumor cells and normal cells at the mRNA level are suitable for use in peptide-based cancer immunotherapy. Among the previously reported CTL-directed antigens, most were ubiquitously expressed in both

tumor cells and normal tissues at the mRNA level, with preferential or exclusive expression in tumor cells at the protein level (2, 4–8), whereas *MRP3* showed preferential expression at the mRNA level (3). However, in our above-mentioned clinical trials of peptide vaccinations for several epithelial cancers, including colon cancer, there were no apparent differences in safety or tumor regression between the peptides from the former group and those from the latter group, and major tumor regression was observed in a few patients with no severe adverse events (21–25).

The HLA-A2 allele is found in 23% of Africans, 53% of Chinese, 40% of Japanese, and 50% of Caucasians (12). With regard to HLA-A2 subtypes, HLA-A0201 is found in the majority of HLA-A2<sup>+</sup> Caucasians and in 45% of HLA-A2<sup>+</sup> Japanese, whereas HLA-A0206 is found in 36% of HLA-A2<sup>+</sup> Japanese (26). PGK1-derived peptide at positions 235 to 243 induced CTL activity in PBMCs from patients with all three HLA-A2 subtypes, whereas AICRT/I at positions 90 to 98 induced CTL activity in PBMCs of HLA-A0201 and HLA-A0206<sup>+</sup> patients. Two peptides (TYMS at positions 189 to 198 and AICRT/I at positions 288 to 296) induced CTL activity in HLA-A0201 and HLA-A0207<sup>+</sup> patients. These results suggest that each of the four peptides has the ability to induce HLA-A2-restricted CTLs reactive to tumor cells, in agreement with our previous observation (8). However, additional studies with more samples with different subtypes will be needed to confirm whether or not each of these four peptides has the ability to induce HLA-A2-restricted and tumor-reactive CTLs from HLA-A2 colon cancer patients irrespective of the HLA-A2 subtype.

The results of these studies showed that four peptides from three proliferation-related proteins had the ability to induce HLA-A2-restricted CTLs reactive to colon tumor cells in PBMCs of colon cancer patients. These results may provide a better understanding of the molecular features of colon cancer antigens and CTL-directed peptides.

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# 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> peptide-specific CTLs reactive to tumor cells from HLA-A33<sup>+</sup> 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<sup>+</sup> 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*<sup>+</sup> 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*<sup>+</sup> 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 <sup>51</sup>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  $^{51}\text{Cr}$  release assay and an IFN- $\gamma$  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 *Sa*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  $\mu\text{L}$  of Lipofectamine (Invitrogen) in 120  $\mu\text{L}$  of Opti-MEM (Invitrogen) for 40 minutes. The COS-7 cells ( $5 \times 10^3$ ) were incubated with 50  $\mu\text{L}$  of the mixture for 6 hours, followed by the addition of 150  $\mu\text{L}$  of RPMI 1640 containing 10% FCS. A total of  $1 \times 10^5$  clones from the LC-1 cDNA library was tested for their ability to stimulate IFN- $\gamma$  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- $\gamma$  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- $\gamma$  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  $^{32}\text{P}$ -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, immunohistochem-

istry 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 ( $1 \times 10^4$  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 ( $1 \times 10^6$  cells) were suspended in Opti-MEM containing 3  $\mu\text{g}/\text{mL}$  human  $\beta_2$ -microglobulin and 10  $\mu\text{g}/\text{mL}$  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 ( $1 \times 10^4$  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 ( $2 \times 10^5$  cells per well) were added and incubated for an additional 18 hours. Levels of IFN- $\gamma$  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 ( $1 \times 10^5$  cells 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- $\gamma$  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- $\gamma$  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- $\gamma$  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  $^{51}\text{Cr}$  release assay (13). For the inhibition test, 20  $\mu\text{g}/\text{mL}$  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.

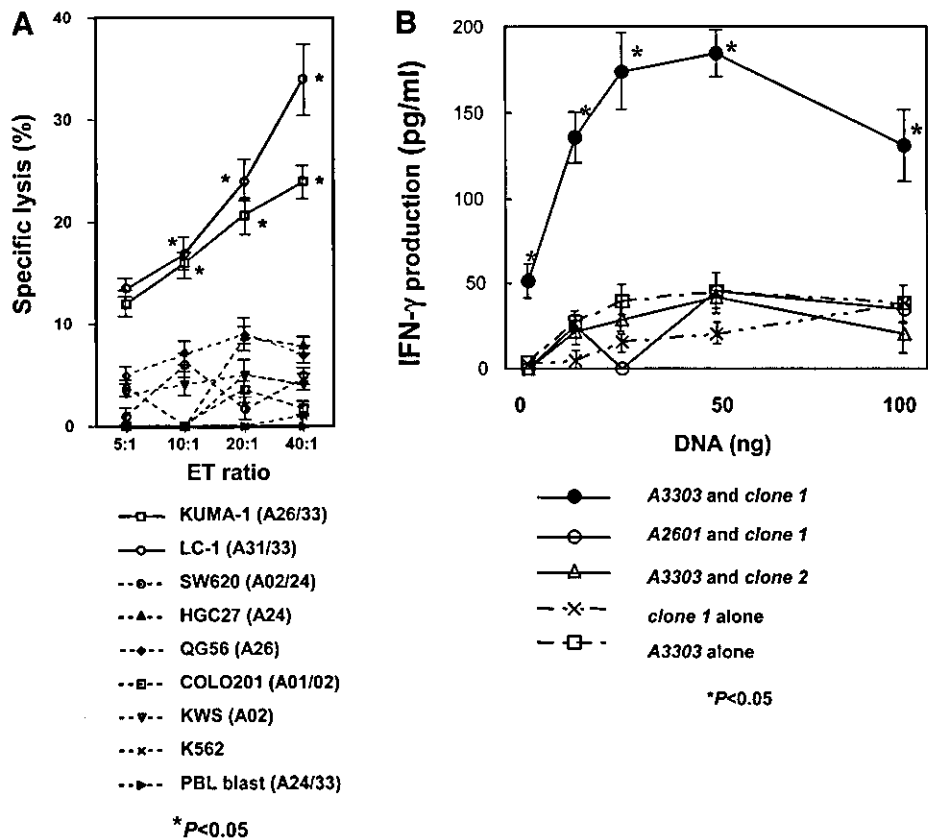
## RESULTS

**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<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> phenotype showed HLA-A33-restricted and tumor-specific CTL activity as measured by <sup>51</sup>Cr release assay. As shown in Fig. 1A, the 850B-CTL1 showed cytotoxicity against LC-1 and KUMA-1 cells but not against any of the HLA-A33<sup>-</sup> tumor cells, COS-7 cells, natural killer target cell line K562, or HLA-A33<sup>+</sup> phytohemagglutinin-activated T cells (phytohemagglutinin blast cells) from the PBMCs of healthy donors. A total of 1 × 10<sup>5</sup> 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. 1B, COS-7 cells transfected with *clone 1* and HLA-A\*3303, but not with *clone 1* 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. 1B). 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. 1B. 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. 2A, a band of ~1 kb was clearly detected in all of the normal tissues tested, except the peripheral blood lymphocytes (Fig. 2A, Lane 12), with specially higher expression in the skeletal muscle (Fig. 2A, Lane 3), kidney (Fig. 2A, Lane 7), liver (Fig. 2A, Lane 8), and placenta (Fig. 2A, Lane 10) and lower expression in the colon (Fig. 2A, Lane 4), small intestine (Fig. 2A, Lane 9), and lung (Fig. 2A, 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. 2B, Lanes 2–5, 12), lung (Fig. 2B, Lanes 6 and 7), head and neck (Fig. 2B, Lane 8), pancreas (Fig. 2B, 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 <sup>51</sup>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.



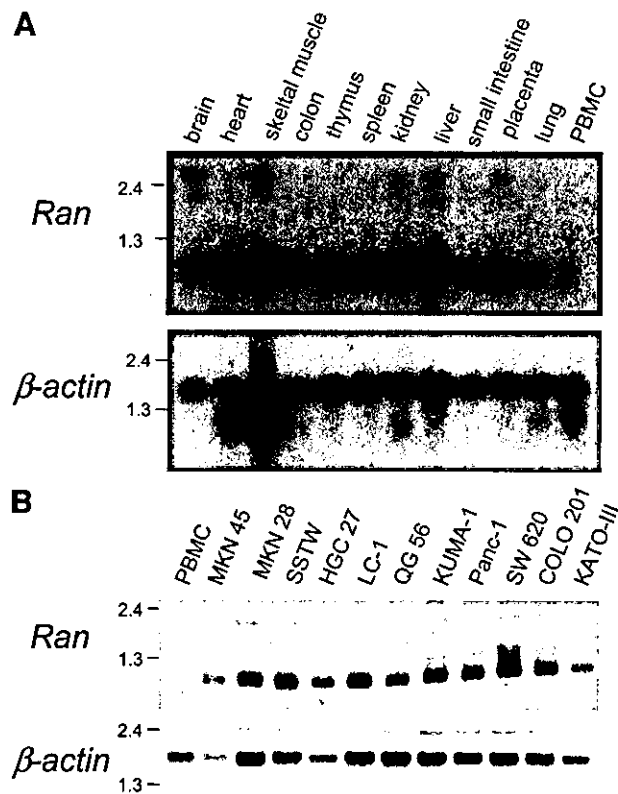


Fig. 2 Expression of *Ran* mRNA. A. The expression of *Ran* or control  $\beta$ -actin mRNA in normal tissues was examined by Northern blot analysis. B. The expression of *Ran* or control  $\beta$ -actin mRNA in a panel of cancer cell lines was examined by Northern blot analysis.

colon (Fig. 2B, 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. 3A–E) and normal tissues (Fig. 3F–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. 3A), colon (Fig. 3B), pancreas (Fig. 3C), 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. 3F, gastric; Fig. 3G, colon; Fig. 3H, pancreas, and; Fig. 3I, 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  $\mu$ mol/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  $\mu$ mol/L, and their ability

to induce IFN- $\gamma$  production by 850B-CTL1 was tested. Four of these peptides, Ran48-56, Ran87-95, Ran97-106, and Ran121-129, induced significant levels of IFN- $\gamma$  production (Fig. 4A) in a dose-dependent manner (Fig. 4B). The optimal concentration of Ran48-56, Ran87-95, and Ran97-106 peptides for loading onto the HLA-A33-transfected C1R cells was 1  $\mu$ mol/L, whereas that of Ran121-129 peptide, which has the lowest binding affinity among the tested 8 peptides (Table 1), was higher than 1  $\mu$ mol/L (>10  $\mu$ mol/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- $\gamma$  (data not shown).

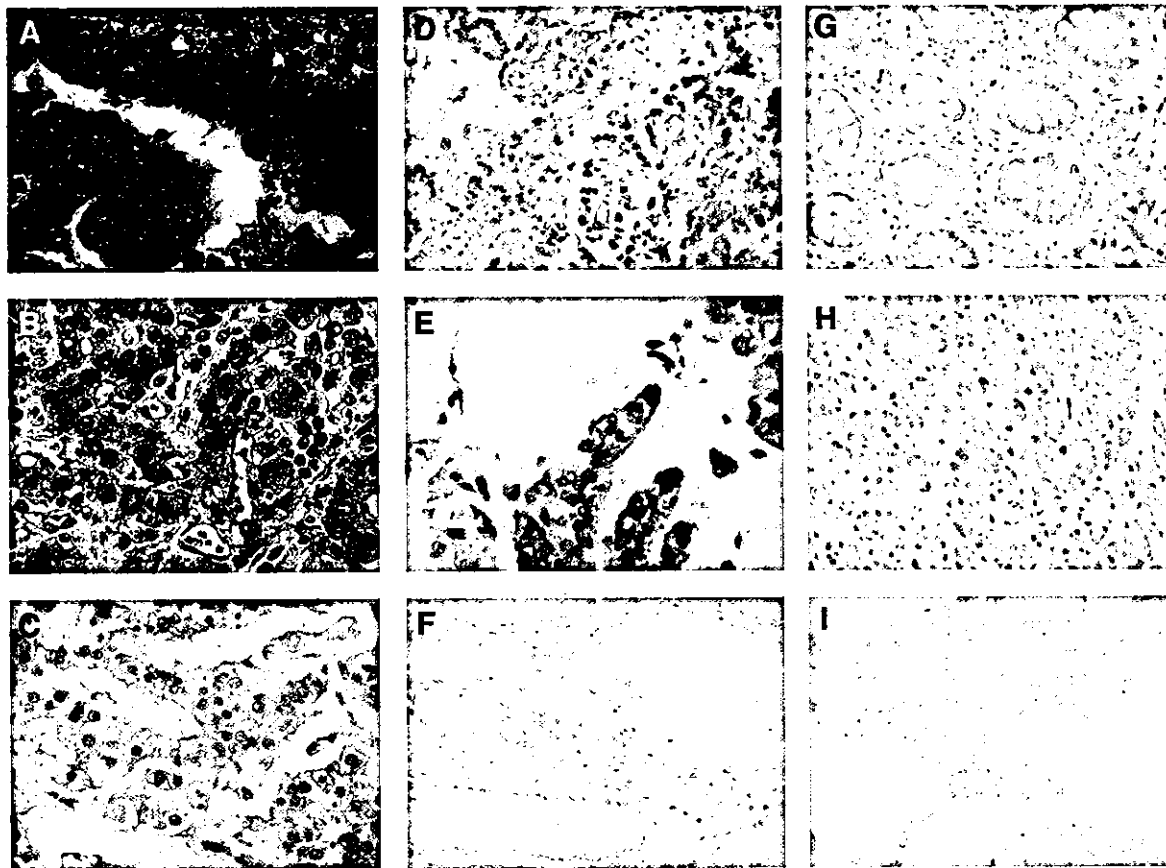
**Induction of CTL by *Ran*-derived Peptides.** The Ran48-56, Ran87-95, Ran97-106, and Ran121-129 peptides were then tested for their ability to induce HLA-A33-restricted and tumor-specific CTLs in the PBMCs of 10 HLA-A33<sup>+</sup> epithelial cancer patients. Ran48-56 and Ran87-95 stimulated PBMCs from 4 and 5 of 10 cancer patients tested to produce significant amounts of IFN- $\gamma$  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 <sup>51</sup>Cr release assay. The PBMCs stimulated with either the Ran48-56 or Ran87-95 peptide showed significant levels of cytotoxicity against the HLA-A33<sup>+</sup> LC-1 tumor cells but not against either HLA-A33<sup>-</sup> QG56 cells or the HLA-A33<sup>+</sup> phytohemagglutinin blast cells in all of the cases in which CTL activities were observed by IFN- $\gamma$  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<sup>+</sup> 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

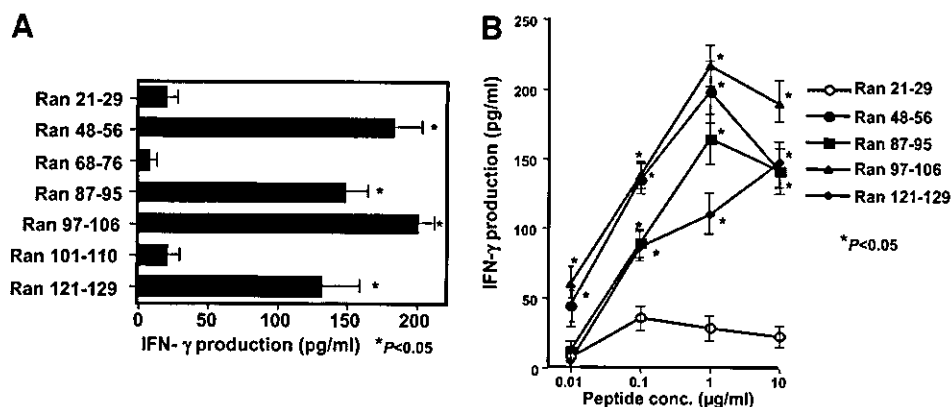
tested at both the mRNA and protein levels. In contrast, regardless of its expression in normal tissues at the mRNA level, it was not enhanced in the surrounding normal cells or tissues. It was also undetectable in normal tissues as far as

**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 (LLGPRPYR)  | 15.6                        |
| Ran 21-29 (TGKTFVKR)     | 15.6                        |
| Ran 48-56 (HPLVFHTNR)    | 14.6                        |
| Ran 68-76 (GQEKFGGLR)    | 14.8                        |
| Ran 87-95 (IIMFDVTSR)    | 14.5                        |
| Ran 97-106 (TYKNVFNWHR)  | 15.0                        |
| Ran 101-110 (VPNWHRLVLR) | 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  $\mu\text{g}/\text{mL}$ . The 850B-CTL1 cells were cultured with the peptide-loaded C1R-A33 for 18 hours, and the culture supernatant was harvested to measure IFN- $\gamma$  using an ELISA. The background of the IFN- $\gamma$  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- $\gamma$  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- $\gamma$  production by the 850B-CTL1 cells was tested. The background of the IFN- $\gamma$  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 phosphatase (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

| Peptide    | Patient no. |            |            |            |        |            |            |            |            |             | Total |
|------------|-------------|------------|------------|------------|--------|------------|------------|------------|------------|-------------|-------|
|            | 1 Lung      | 2 Cervical | 3 Uterus   | 4 Prostate | 5 Lung | 6 Prostate | 7 Prostate | 8 Gastric  | 9 Prostate | 10 Prostate |       |
| RAN48-56   | 44          | <u>246</u> | <u>123</u> | <u>103</u> | 38     | 27         | 67         | <u>134</u> | 17         | 23          | 4/10  |
| RAN87-95   | 51          | 29         | 4          | <u>157</u> | 15     | <u>159</u> | <u>118</u> | <u>95</u>  | <u>114</u> | 16          | 5/10  |
| RAN97-106  | 35          | 102        | 21         | 87         | 21     | <u>133</u> | 53         | 44         | 39         | 75          | 1/10  |
| RAN121-129 | <u>161</u>  | 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- $\gamma$  production by the effector PBMCs in response to the C1R-A33 cells prepulsed with the corresponding peptide. The background IFN- $\gamma$  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.

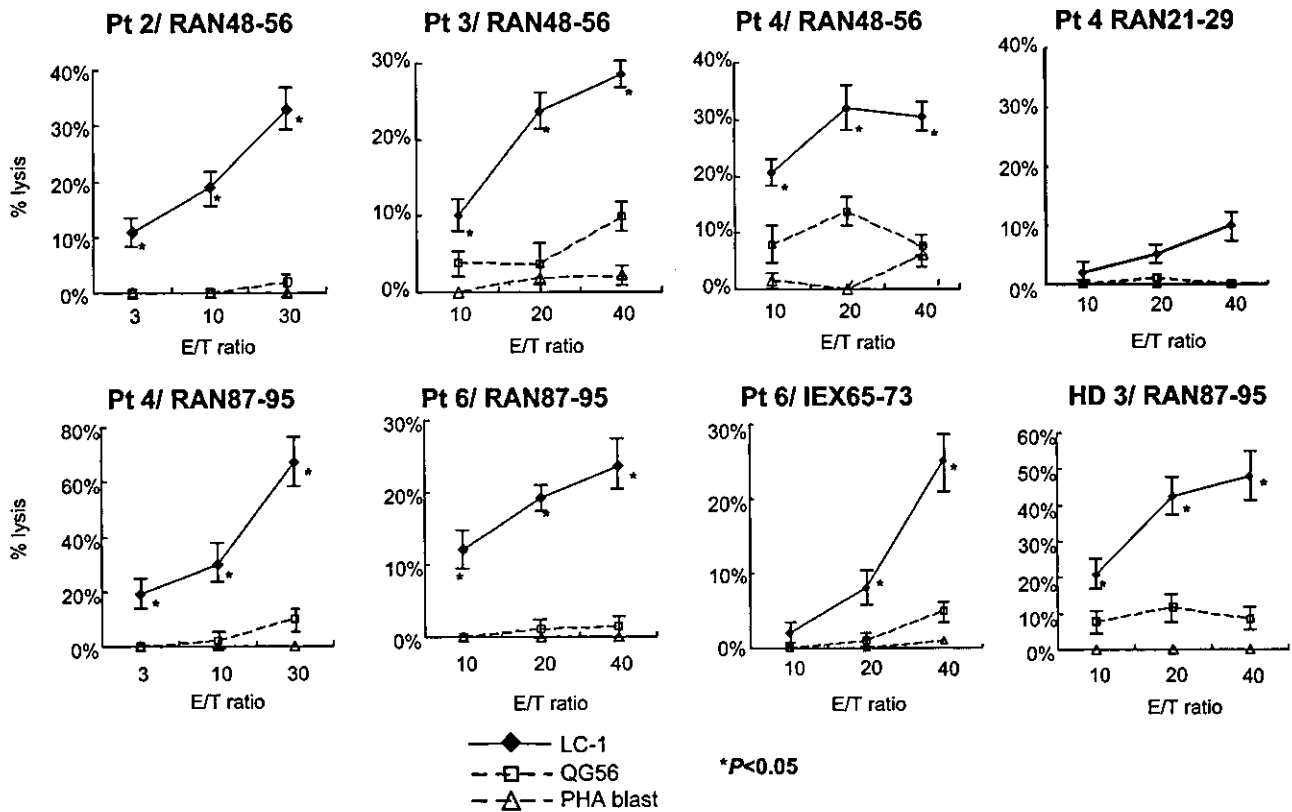


Fig. 5 Cytotoxicity of the peptide-induced CTLs. Peptide-specific CTLs were induced by the Ran-derived peptides, Ran48-56 and Ran87-95, in the PBMCs from cancer patients. The cytotoxic activity of the peptide-induced CTLs against the LC-1 (HLA-A33<sup>+</sup>) and QG56 (HLA-A33<sup>-</sup>) tumor cells was measured by a 6-hour <sup>51</sup>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.

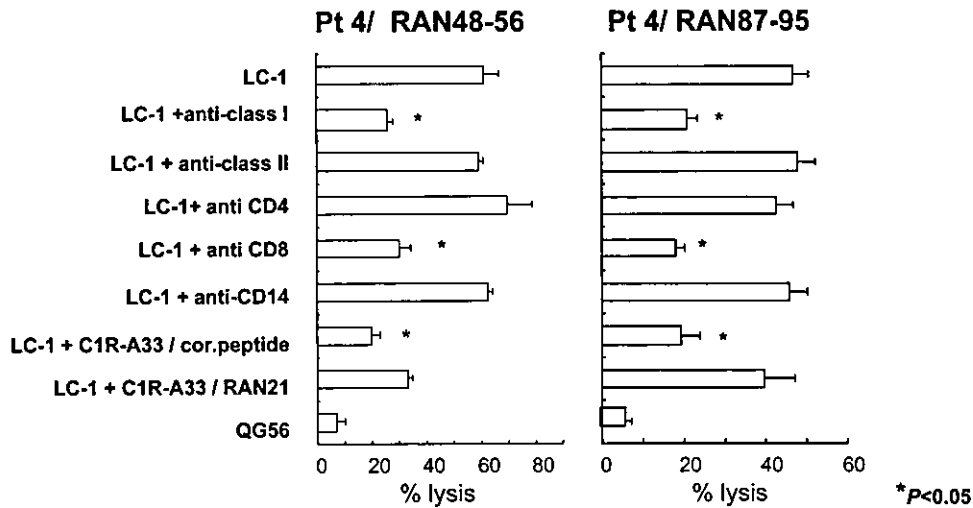


Fig. 6 Inhibition and competition assays. For the inhibition test, the peptide-stimulated PBMCs were tested for their restriction and for the peptide specificity of cytotoxicity against LC-1 (HLA-A33<sup>+</sup>) by a standard 6-hour <sup>51</sup>Cr release assay in the presence or absence of 20  $\mu$ g/mL anti-HLA class I (W6/32, IgG2a), anti-HLA class II (H-DR-1, IgG2a), anti-CD4 (Nu-Th1, 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 <sup>51</sup>Cr release assay at a cold-to-hot target cell ratio of 10 to 1. The 6-hour <sup>51</sup>Cr release assay was performed at an E:T ratio of 10 to 1. Values represent the means  $\pm$  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<sup>+</sup> cancer patients.

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## Immunological Evaluation of Individualized Peptide Vaccination With a Low Dose of Estramustine for HLA-A24<sup>+</sup> HRPC Patients

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**BACKGROUND.** The safety, toxicity, and immunological response of individualized peptide vaccination or human leukocyte antigen (HLA)-A24<sup>+</sup> hormone refractory prostate cancer (HRPC) patients in combination with a low dose of estramustine were evaluated.

**METHODS.** Sixteen patients with HLA-A24<sup>+</sup> HRPC were enrolled in the phase I/II study. Conducted immune monitorings for those patients were peptide-specific cytotoxic T lymphocyte (CTL) precursor analysis by interferon- $\gamma$  production and peptide-reactive immunoglobulin G (IgG) by an enzyme-linked immunosorbent assay. Clinical responses and quality of life (QOL) outcomes using a self-reported patient questionnaire were also evaluated.

**RESULTS.** Vaccinations were well tolerated, but all patients developed grade 1 or 2 local redness and swelling at the injection site. There was no significant immunosuppression in most cases when the peptide and a half dose (280 mg/day) of estramustine were administered. Augmentation of peptide-specific CTL precursors or peptide-specific IgG was observed in 10 of 14 or 7 of 14 patients at 12 weeks (peptide vaccination alone), and in 6 of 8 or 10 of 12 patients at 24 weeks (during the combination therapy), respectively. All 13 patients treated, with the combination therapy, showed a decrease of serum prostate-specific antigen (PSA) level from the baseline, including six patients with a serum PSA level decrease of  $\geq 50\%$ . QOL outcomes were not deteriorated during the treatment.

**CONCLUSION.** These results might encourage the further evaluation of the combination of peptide vaccination and a low dose of estramustine phosphate for HLA-A24<sup>+</sup> HRPC patients. *Prostate* 9999: 1–12, 2004. © 2004 Wiley-Liss, Inc.

**KEY WORDS:** prostate cancer; immunotherapy; cancer vaccine; estramustine phosphate

### INTRODUCTION

Metastatic prostate cancer is primarily treated by hormonal therapy. However, all such treated patients eventually develop disease refractory to androgen suppression as manifested by increasing serum prostate-specific antigen (PSA) levels, progressive disease on radiographic imaging, and ultimately, symptomatic deterioration. Although, the median survival duration of patients with hormone-refractory prostate cancer

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(HRPC) has been considerably prolonged [1], the improvement is unsatisfactory. Therefore, novel therapeutic agents for the treatment of HRPC are required.

Recent advances in tumor immunology have resulted in identification of a number of antigens and their peptides recognized by tumor-reactive and human leukocyte antigen (HLA) class I-restricted cytotoxic T lymphocytes (CTLs) [2–5]. Anti-tumor vaccines have emerged as a promising therapeutic approach [6–8], whereas, their clinical responses have been limited. In order to develop new treatment modality for HRPC patients, we recently devised a new regime of peptide-based vaccination; the measurement of pre-existing peptide-specific CTL precursors reactive to many kinds of vaccine candidates, followed by administration of only CTL-reactive peptides (individualized peptide vaccination) [9,10]. In addition, we recently reported a benefit of the combination of this type of peptide vaccination and a low dose (280 mg/day) of estramustine phosphate in patients with metastatic HRPC who had received the prior vaccination [11]. However, CTL epitope peptides used in the trial were derived from widely expressed epithelial cancer-related antigens. Therefore, we further attempted to identify prostate-related antigen-derived CTL epitope peptides immunogenic in HRPC patients [12–14], because these antigens are promising target molecules in specific immunotherapy for prostate cancer patients [15].

In this phase I/II trial, HLA-A24<sup>+</sup> patients with HRPC were treated with the combination of peptides, from 16 candidates derived from newly determined prostate cancer-related antigens [12–14] and the previously determined epithelial cancer-related antigens [16–19], and a low dose of estramustine phosphate. The feasibility, toxicity, clinical and immunological responses of this combination therapy were evaluated by analyzing serially measured serum PSA, computed tomography (CT) imaging, clinical bone scan recordings, peptide-specific CTL precursors by interferon (IFN)- $\gamma$  release assay and peptide-reactive immunoglobulin G (IgG) by an enzyme-linked immunosorbent assay. Quality of life (QOL) outcomes using a self-reported patient questionnaire were also evaluated during the treatment.

## PATIENTS AND METHODS

### Patients

Patients with HLA-A24<sup>+</sup> and pathologically confirmed adenocarcinoma of the prostate were entered into a phase I/II study in which patients were treated by a regime of the individualized peptide vaccination followed by a combination therapy with oral estramustine phosphate after disease progression when the

patients were evaluated as having progression disease (PD) by peptide vaccination alone. Eligibility required progression of PD after androgen ablation and second line hormone therapy, as defined by the following criteria. Disease progression was defined by at least one of three criteria: two consecutive 25% increases from baseline PSA levels at least 2 weeks apart, a greater than 25% increase in bidimensionally measurable soft tissue metastases or the appearance of new foci on radio-nuclide bone scans. To control for the effects of androgen ablation, luteinizing hormone-releasing hormone therapy was maintained. Antiandrogen therapy was discontinued for at least 4 weeks before enrollment for patients receiving flutamide and 6 weeks for those receiving bicalutamide. Patients were required to wait at least 4 weeks for study entry after the completion of prior chemotherapy, radiation therapy, or a change in hormonal therapy. Other eligibility included an Eastern Cooperative Oncology Group performance status of 0 or 1, age 79 years or less, granulocyte count greater than 3,000/mm<sup>3</sup>, hemoglobin greater than 10 g/dl, platelets greater than 100,000/mm<sup>3</sup>, bilirubin of less than 1.5 mg/dl, and creatinine less than 1.4 mg/dl. Negative serologic tests for hepatitis B and hepatitis C were required. Patients with a serious illness or an active secondary malignancy within the prior 5 years were excluded from study entry. Exclusion criteria also included evidence of immunosuppression or autoimmune disease. This study was approved by the Kurume University School of Medicine ethics committee to be conducted according to good research practice guidelines (approved number #2136), and all patients were required to give written informed consent prior to study entry.

### Peptide Selection

All patients received more than six vaccinations of four peptides from 16 candidates (SART1<sub>690-698</sub>, SART2<sub>93-101</sub>, SART2<sub>161-169</sub>, SART2<sub>899-907</sub>, SART3<sub>109-118</sub>, SART3<sub>315-323</sub>, Lck<sub>208-216</sub>, Lck<sub>486-487</sub>, Lck<sub>488-497</sub>, ART1<sub>170-179</sub>, prostate acid phosphate (PAP)<sub>213-221</sub>, PSA<sub>152-160</sub>, PSA<sub>248-257</sub>, prostate-specific membrane antigen (PSMA)<sub>624-632</sub>, multidrug resistance-associated protein (MRP)3<sub>503-511</sub> and MRP3<sub>1293-1302</sub> at 2 week intervals. These peptides were prepared under conditions of Good Manufacturing Practice by a Multiple Peptide System (San Diego, CA). All of these peptides have the ability to induce HLA-A24-restricted and tumor-specific CTL activity in peripheral blood mononuclear cells (PBMCs) of the cancer patients [9–19]. Before the first vaccination, 30 ml of peripheral blood was obtained, and PBMCs and plasma were isolated by means of Ficoll-Conray density gradient centrifugation. Both anti-peptide IgG in serum and

peptide-specific CTL precursors in PBMCs were measured as reported previously (Mine et al., 2004). To measure peptide-specific CTL precursors, PBMCs ( $1 \times 10^5$  cells/well) were incubated with  $10 \mu\text{M}$  of each peptide in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark) in  $200 \mu\text{l}$  of culture medium. On the 12th day of the culture, 24 hr after the last stimulation, these cells were harvested, washed three times, and then tested for their ability to produce IFN- $\gamma$  in response to C1R-A2402 cells preloaded with either a corresponding peptide or HIV peptide (RYLRQQLGI) as a negative control. After incubation for 18 hr, the supernatants ( $100 \mu\text{l}$ ) were collected, and the amounts of IFN- $\gamma$  were measured using ELISA (limit of sensitivity:  $10 \text{ pg/ml}$ ). All experiments were performed in four different wells with duplicate assays. Pre-vaccination PBMCs were provided for screening of the CTL precursors reactive to 16 peptides for HLA-A24 positive patients for different wells with duplicate assays in each well, and the results of each well were classified into four groups in accordance with the *P* values (by two-tailed Students' *t* test) and the amounts of IFN- $\gamma$  (a mean value response to a corresponding peptide minus that to an HIV peptide) as follows: Armed response (Ar):  $P \leq 0.01$  and  $500 \leq \text{net}$ ; A level of response (A):  $P \leq 0.05$  and  $50 \leq \text{net}$ ; B:  $P \leq 0.05$  and  $25 \leq \text{net} < 50$ ; C:  $0.05 < P \leq 0.1$  and  $50 \leq \text{net}$ . We previously reported that IgG specific to each of those peptides can be frequently detected in pre- and post-vaccination sera, and the levels of anti-peptide IgG is a laboratory marker to predict clinical responses to the individualized peptide vaccination with good relation to overall survival [20].

Therefore, peptides were chosen based upon evaluation of both levels of anti-peptide IgG in plasma and CTL precursors in PBMCs: for first selection, a peptide with high titer of anti-peptide IgG and a high level (Ar, A, B, and C ranks shown above) of responses in CTL precursors; for second selection, a peptide with high titer of anti-peptide IgG; for third selection, a peptide with a high level of responses in CTL precursors; for no selection, a peptide with low titer of anti-peptide IgG and a low level (less than C rank) of responses in CTL precursors.

An ELISA was used to detect the serum IgG levels specific for the peptides administrated, as reported previously [21,22]. In brief, the peptides immobilized plate ( $20 \mu\text{g/well}$ ) was blocked with Block Ace (Yukijirushi, Tokyo, Japan), washed, and  $100 \mu\text{l/well}$  of serum samples diluted with 0.05% Tween 20-Block Ace were added to the plate. After 2 hr incubation, the plate was washed and further incubated for 2 hr with a 1:1,000-diluted rabbit anti-human IgG (DAKO, Glostrup, Denmark). The plate was washed and then  $100 \mu\text{l}$  of 1:100-diluted goat anti-rabbit Ig-conjugated

horseradish peroxidase-dextran polymer (Envision, DAKO) was added to each well, and the plate was incubated for 40 min. After washing,  $100 \mu\text{l/well}$  of tetramethyl-benzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by an addition of 1 M phosphoric acid. To estimate peptide-specific IgG levels, the optical density values of each sample were compared with those of serially diluted standard serum samples, and the values were shown as optical density. To confirm the specificity of IgG to the peptide,  $100 \mu\text{l}$  of the samples were incubated in the peptide-coated wells to absorb peptide-specific IgG and the levels of peptide-specific IgG in the resulting samples were determined.

### Vaccination and Combination Therapy

The peptide vaccination schedule was as follows. For the skin test,  $10 \mu\text{g}$  of each selected peptide for up to four peptides were independently injected intradermally with a tuberculin syringe with a 27-gauge needle. Immediate- and delayed-type hypersensitivity (DHT) reactions were determined at 20 min and 24 hr after the skin test, respectively. A positive skin-test reaction was defined as  $>30\text{-mm}$  diameter erythema and induration when saline was a negative control for the assessment of hypersensitivity. If immediate-type hypersensitivity was negative, the peptide was injected. A peptide was added in a 1:1 volume to Montanide ISA-51, then mixed in a Vortex mixer. The resulting emulsion was injected. Before the combination therapy,  $3 \text{ mg/ml}$  of each peptide was injected subcutaneously in the lateral thigh of each patient a total of six times at 2-week intervals. During the combination therapy,  $1 \text{ mg/ml}$  of each peptide was injected at 2- to 4-week intervals.

Estramustine phosphate was administered orally as a  $140 \text{ mg}$  capsule, 1 capsule twice daily, for a total daily dose of  $280 \text{ mg}$  as severe immunosuppression in the combination therapy was avoided by reducing estramustine phosphate to  $280$  from  $560 \text{ mg/day}$  in our previous study [11].

### Immunological Monitoring

For evaluation of immune responses during immunotherapy alone and combination with estramustine phosphate, peptide-specific CTL precursors in PBMCs, and serum levels of peptide-specific antibodies were measured after third vaccination and following every 6th vaccination.

### Clinical Monitoring

Patients were observed until disease death or intolerance, or consent was withdrawn. Clinical and laboratory assessments were performed at each visit,

and patients were questioned about adverse events, their severity and frequency. The severity of adverse events was scored according to the National Cancer Institute (NCI) Toxicity Criteria (Version 2, 1998). Serum PSA were measured every 2 weeks during the treatment. Bone scans and CT scans of the abdomen were performed every 3 months during this study. The metastatic findings on bone scans were assessed by the extent of the disease using the percentage of positive area on the bone scan (% PABS) [23]. Clinical response was determined by both changes in PSA levels and by imaging studies in patients with measurable disease. PSA response was defined as two consecutive measurements at least 2 weeks apart that showed a 50% or greater decrease from baseline PSA levels (PR: partial response) or normalization of the PSA level (CR: complete response). Time of PSA progression was registered at the time of the first of two consecutive PSA levels 25% above the baseline. Standard definitions were used for response and progression of measurable and evaluable disease. For patients with bidimensionally measurable disease, a CR was defined as the disappearance of all target lesions for at least 4 weeks; a PR was defined as a  $\geq 50\%$  decline in bidimensionally measurable disease, and a minor response was defined as a reduction between 25 and 50%. For a response of bone metastasis, a CR was then defined as the disappearance of all positive areas on bone scans. A PR was defined as a 50% or greater decrease in the % PABS, and PD was defined as an increased number of positive sites, increased intensity of the existing lesions or the two findings observed concurrently.

QOL outcomes of patients during this treatment were evaluated at pre-vaccination, and 3rd, 6th, and 12th vaccination using a Japanese version of the Functional Assessment of Cancer Therapy (FACT) subscale, FACT-P (for prostate cancer) [24]. This Japanese version of the questionnaire was validated in Japan to be as useful as the original version [25]. The FACT-P questionnaire consist of five factors including physical well being (seven items), social/family well being (eight items), emotional well being (six items), functional well being (seven items), and prostate cancer scale (12 items). QOL outcomes were separately assessed by the percentage of each scale.

#### Statistical Methods

Data were analyzed using commercially available computer software. Cancer related survival was defined as the time from the registration of the phase I/II study to disease caused death. The Kaplan-Meier method was used to estimate cancer related survival and groups were compared using a log-rank test.

Student's *t*-test and the chi-square test were used to compare quantitative and categorical variables, respectively. Tests results were considered significant at  $P < 0.05$ . The software used for the statistical analysis of Student's *t*-test is Stat View 5.0.

## RESULTS

### Patient Characteristics

Between March, 2002 and January, 2003, 16 patients positive for HLA-A24 with metastatic HRPC entered into the phase I/II study. The median age of the patients was 69 (range, 54–78) years and the median Eastern Cooperative Oncology Group performance status was 0 (range, 0–1). All 16 patients had progressed on initial hormonal therapy including combined androgen blockade (leuprolide plus flutamide or bicalutamide in 15 patients and bilateral orchiectomy with bicalutamide in 1 patient). Of all patients treated, nine patients had received prior estramustine-based therapy as a second line treatment: treatment with estramustine (seven patients) or estramustine combined with etoposide (two patients). The remaining six patients had another second line hormone therapy with radiation therapy for local control (three patients) or a high dose of intravenous diethylstilbestrol diphosphate therapy (three patients). The median baseline PSA was 43.1 (range, 6.3–2,269.5) ng/ml. At study entry, 13 patients had metastases of the bone including four patients with lymph node involvement, and three patients had lymph node involvement (two patients) or liver (one patient) metastasis. All patients received more than six vaccinations of four peptides from 16 candidates derived from SART1, SART2, SART3, Lck, ART1, PAP, PSA, PSMA, and MRP3 at 2 week intervals. Thirteen patients were followed by the combined peptide vaccination and a low dose of estramustine phosphate when the disease progressed after at least the 3rd peptide vaccination. The remaining three patients had only vaccine therapy because of quick disease progression to death. Table I summarizes the clinical characteristics for the 16 patients with HRPC in this study.

### Vaccination and Toxicity

All patients were available for toxicity analyses and 13 of the 16 patients were available for clinical responses. The median number of vaccinations of the patients was 12 (range, 6–19). Thirteen patients received combination therapy of vaccine plus estramustine phosphate and three patients received vaccine therapy alone. All 16 patients were evaluated for all common toxicities, and the overall toxicities are shown in Table II. One case (patient 4) of grade 2 cerebral infarction was observed during the combination therapy.

TABLE I. Patient Characteristics

|   | Vaccine alone | Vaccine plus estramustine phosphate | Total      |
|---|---------------|-------------------------------------|------------|
| Number of patients                      | 3             | 13                                  | 16         |
| Age (year)                              |               |                                     |            |
| Median                                  | 69            | 69                                  | 69         |
| Range                                   | 61-73         | 54-78                               | 54-78      |
| Performance status <sup>a</sup>         |               |                                     |            |
| 0                                       | 1             | 12                                  | 13         |
| 1                                       | 2             | 1                                   | 3          |
| Serum PSA level (ng/ml)                 |               |                                     |            |
| Median                                  | 92.5          | 34.8                                | 43.1       |
| Range                                   | 25.1-2269.5   | 6.3-339.6                           | 6.3-2269.5 |
| Gleason score (n)                       |               |                                     |            |
| 7                                       | 0             | 6                                   | 6          |
| 8                                       | 1             | 3                                   | 4          |
| 9                                       | 2             | 4                                   | 6          |
| Site of metastases (n)                  |               |                                     |            |
| Bone only                               | 2             | 7                                   | 9          |
| Bone and nodal/organ                    | 1             | 3                                   | 4          |
| Nodal/organ                             | 0             | 3                                   | 3          |
| Prior use of estramustine phosphate (n) |               |                                     |            |
| Yes                                     | 2             | 7                                   | 9          |
| No                                      | 1             | 6                                   | 7          |

<sup>a</sup>Performance status by ECOG score.

This patient, who was diagnosed as having slight cerebral infarction by CT examination after fainting, did not need treatment, and combination therapy was continued without any other major toxicities. The most common toxicities were dermatologic reactions at the injection site of the vaccination in all cases. All 16 dermatologic reactions were scored as grade 1 or 2 using National Cancer Institute common toxicity criteria. Seven patients complained of anorexia, six patients complained of bone pain, and two patients developed grade 2 hematuria. There was no treatment related to

hematologic, hepatic, or renal toxicity. In addition, there was no clinical evidence of autoimmune reactions as determined by symptoms, physical examination, or laboratory tests.

#### Vaccinated Peptides and Immune Responses

Before the peptide vaccination, peptide-specific CTL precursors were examined, and were detectable in 14 of 16 patients with a median positive number of 1.5 peptides (range, 0-4 peptides) per patient (Table III). In

TABLE II. Adverse Events

| Toxicity <sup>a</sup> | Vaccine alone |    |    |    | Vaccine plus estramustine phosphate |    |    |    | Total |
|-----------------------|---------------|----|----|----|-------------------------------------|----|----|----|-------|
|                       | G1            | G2 | G3 | G4 | G1                                  | G2 | G3 | G4 |       |
| Dermatologic          | 3             |    |    |    | 12                                  | 1  |    |    | 16    |
| Anorexia              | 1             |    |    |    | 6                                   |    |    |    | 7     |
| Bone pain             |               |    |    |    | 1                                   | 5  |    |    | 6     |
| Hematuria             |               | 1  |    |    |                                     | 1  |    |    | 2     |
| Edema                 |               |    |    |    | 2                                   |    |    |    | 2     |
| Cerebral infarction   |               |    |    |    |                                     | 1  |    |    | 1     |
| Anemia (Hb <8 mg/dl)  |               | 1  |    |    |                                     |    |    |    | 1     |
| Fatigue               | 1             |    |    |    |                                     |    |    |    | 1     |

<sup>a</sup>Toxicities based on the National Cancer Institute common toxicity scale (Version 2). Some patients had more than one toxic reaction.



TABLE III. Immune Response During the Therapy

| Patient number | Peptide   | Cellular response to peptide <sup>a</sup> |         | Anti-peptide IgG <sup>b</sup> |     | DTH induction | Therapy <sup>c</sup> | Best clinical response | PR duration (M) | Follow up (M) | Prognosis |
|----------------|-----------|---|---------|-------------------------------|-----|---------------|----------------------|------------------------|-----------------|---------------|-----------|
|                |           | Pre                                       | 6th     | Pre                           | 6th |               |                      |                        |                 |               |           |
| 1              | SART1 690 | 449                                       | 35      | -                             | -   | -             | Combination          | PR <sup>d</sup>        | 6               | 17            | Alive     |
|                | SART3 109 | 0   | 681.5   | +                             | +   | +(3)          |                      |                        |                 |               |           |
|                | PAP213    | 0   | 392.3*  | +                             | +   | -             |                      |                        |                 |               |           |
| 2              | PSA248    | 19.5                                      | 100     | +                             | +   | -             | Combination          | PR                     | 3               | 10            | Death     |
|                | SART293   | 22.8                                      | 376.8   | +                             | +   | -             |                      |                        |                 |               |           |
|                | SART3 109 | 14.3                                      | 25      | +                             | +   | -             |                      |                        |                 |               |           |
|                | PAP213    | 0   | 46.8    | +                             | ++  | -             |                      |                        |                 |               |           |
|                | PSA248    | 28.3                                      | 505.8   | +                             | +   | -             |                      |                        |                 |               |           |
| 3              | SART3 109 | 26  | 27.3    | -                             | ++  | -             | Combination          | -                      | -               | 16            | Alive     |
|                | SART3 315 | 47.8                                      | 39.3    | -                             | +   | -             |                      |                        |                 |               |           |
|                | kk208     | 34.8                                      | 0       | -                             | -   | -             |                      |                        |                 |               |           |
|                | PSA152    | 34.5                                      | 34.8    | -                             | -   | -             |                      |                        |                 |               |           |
|                | SART3 315 | 4   | 12.8    | +                             | +   | -             |                      |                        |                 |               |           |
| 4              | PAP213    | 30.8                                      | 850*    | +                             | +   | -             | Combination          | PR                     | 10              | 16            | Alive     |
|                | PSA248    | 12.8                                      | 303.5   | +                             | ++  | -             |                      |                        |                 |               |           |
|                | PSM264    | 100                                       | 167     | +                             | +   | -             |                      |                        |                 |               |           |
|                | SART3 109 | 12.3                                      | 534**   | +                             | +   | +(5)          |                      |                        |                 |               |           |
|                | kk208     | 5.8                                       | 676     | +                             | -   | +(5)          |                      |                        |                 |               |           |
| 5              | kk488     | 29.8                                      | 99      | +                             | -   | +(5)          | Combination          | -                      | -               | 9             | Death     |
|                | PSA248    | 14.3                                      | 149.8   | +                             | +   | +(5)          |                      |                        |                 |               |           |
|                | SART3 109 | 5.3                                       | 170.8   | +                             | +   | +(5)          |                      |                        |                 |               |           |
|                | PAP213    | 5.8                                       | 0       | +                             | +   | -             |                      |                        |                 |               |           |
|                | PSA152    | 39.5                                      | 0       | +                             | +   | -             |                      |                        |                 |               |           |
| 6              | PSA248    | 6.5                                       | 0       | +                             | +   | -             | Vaccine              | PR                     | 3               | 7             | Death     |
|                | SART2 93  | 125                                       | 162.5   | -                             | -   | -             |                      |                        |                 |               |           |
|                | SART3 109 | 5.5                                       | 750**   | +                             | +   | -             |                      |                        |                 |               |           |
|                | PAP213    | 0   | 186.8** | -                             | +   | +(6)          |                      |                        |                 |               |           |
|                | PSA248    | 18.5                                      | 9.3     | +                             | +   | -             |                      |                        |                 |               |           |
| 7              | SART1 690 | 0   | 0       | +                             | +   | -             | Combination          | PR                     | 5               | 12            | Death     |
|                | SART3 109 | 0   | 610.8*  | +                             | +   | -             |                      |                        |                 |               |           |
|                | SART3 315 | 0   | 154**   | -                             | +   | -             |                      |                        |                 |               |           |
|                | PSA248    | 0   | 0       | +                             | ++  | -             |                      |                        |                 |               |           |
|                | SART1 690 | 250                                       | 81.8    | -                             | +   | -             |                      |                        |                 |               |           |
| 8              | PAP213    | 375                                       | 36.5    | -                             | +   | -             | Combination          | -                      | -               | 15            | Alive     |
|                | PSA152    | 250                                       | 18.8    | -                             | +   | -             |                      |                        |                 |               |           |
|                | PSA248    | 375                                       | 21.3    | -                             | ++  | -             |                      |                        |                 |               |           |
|                | SART1 690 | 250                                       | 81.8    | -                             | +   | -             |                      |                        |                 |               |           |
|                | PAP213    | 375                                       | 36.5    | -                             | +   | -             |                      |                        |                 |               |           |
| 9              | PSA152    | 250                                       | 18.8    | -                             | +   | -             | Combination          | -                      | -               | 14            | Alive     |
|                | PSA248    | 375                                       | 21.3    | -                             | ++  | -             |                      |                        |                 |               |           |
|                | SART3 109 | 0   | 0       | +                             | +   | -             |                      |                        |                 |               |           |



addition, anti-peptide IgGs were also detectable in 14 of 16 patients with a median positive number of three peptides (0–4 peptides) per patient. Four peptides at maximum were selected for the injection to each patient and the results of the selection were listed in Table III. The most frequently selected peptide was PSA<sub>248–257</sub> (13/16), followed by PAP<sub>213–221</sub> (11/16), SART3<sub>109–118</sub> (11/16), SART3<sub>315–323</sub> (6/16), PSA<sub>152–160</sub> (5/16), Lck<sub>488–497</sub> (5/16), SART1<sub>690–698</sub> (3/16), SART2<sub>93–101</sub> (3/16), and Lck<sub>208–216</sub> (3/16). PSMA<sub>624–628</sub> and SART2<sub>161–169</sub> were selected in each patient, while the remaining five peptides derived from SART2, Lck, ART1, and MRP3 were not selected in these patients. As shown in Table III, augmentation of peptide-specific CTL precursors to at least one peptide among the four

vaccinated peptides was observed in 8 of 14 patients at 12 weeks (after the 6th vaccination) when the total sum of IFN production from the quadruplicate assays provided for the statistical analysis. This type of augmentation was observed in 10 of the 14 patients when the results of each well were provided for the statistical analysis (data not shown). Augmentation of peptide-specific CTL precursors during the combination therapy was observed in 10 of 12 patients at 24 weeks after the initiation of the vaccination when the results of each well were provided for the statistical analysis, and representative results of seven cases are shown in Figure 1. Figure 2 demonstrates serial changes of IgG levels specific for the peptides administered to each patient. DTH response was observed in 4 of 16 patients,

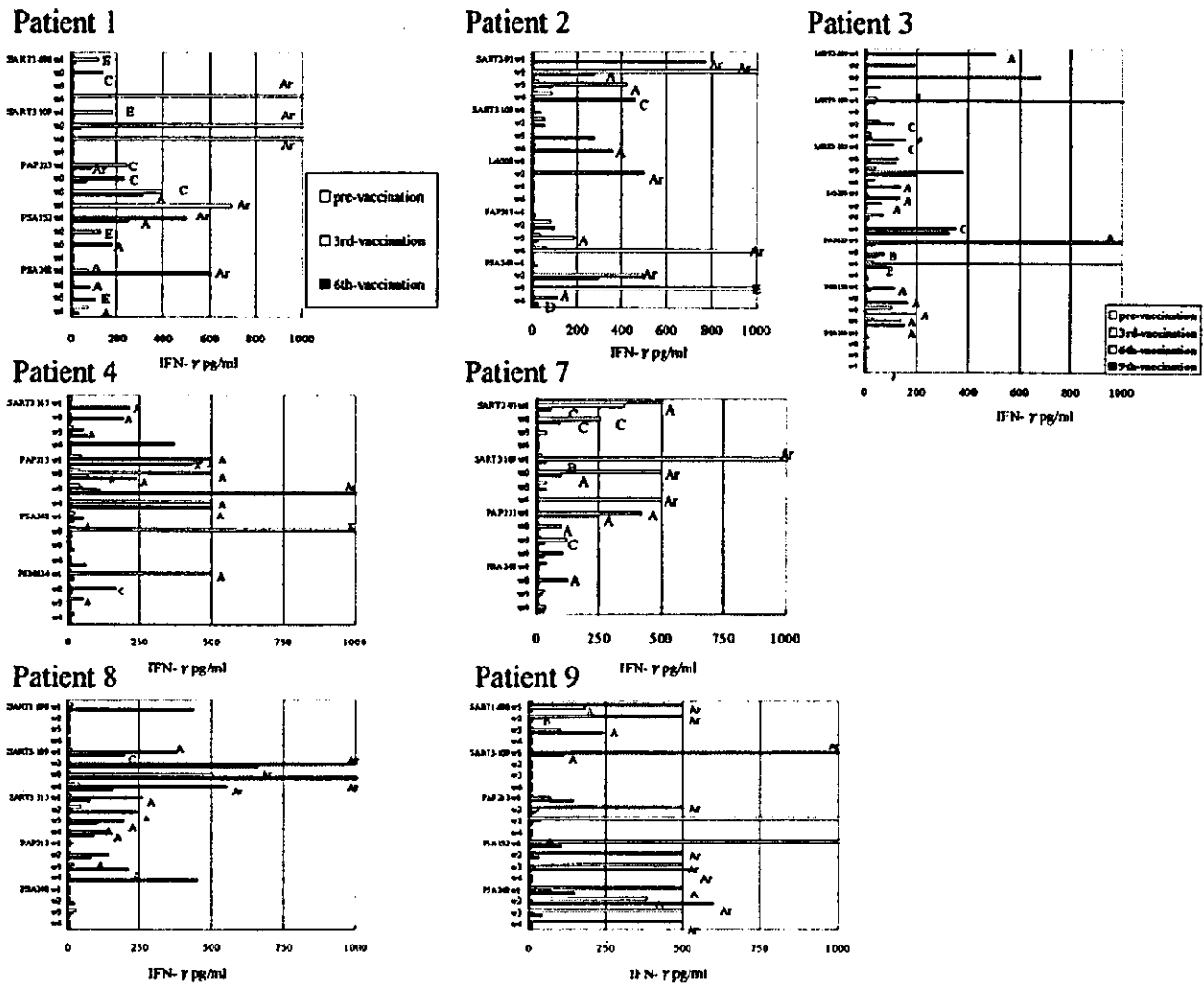


Fig. 1. <sup>Q3</sup> Representative results of peptide-specific CTL precursors in PBMCs of pre- and post-vaccinations. Four sets of columns in each peptide indicate the results of four well cultures and the value of each well was evaluated based on the following criteria. Armed response (Ar):  $P \leq 0.01$  and  $500 \leq \text{net value}$  (the amount of IFN- $\gamma$  in response to the corresponding peptide minus that in response to HIV peptide); A level of response (A):  $P \leq 0.05$  and  $50 \leq \text{net}$ ; (B)  $P \leq 0.05$  and  $25 \leq \text{net} < 50$ ; (C)  $0.05 < P \leq 0.1$  and  $50 \leq \text{net}$ .

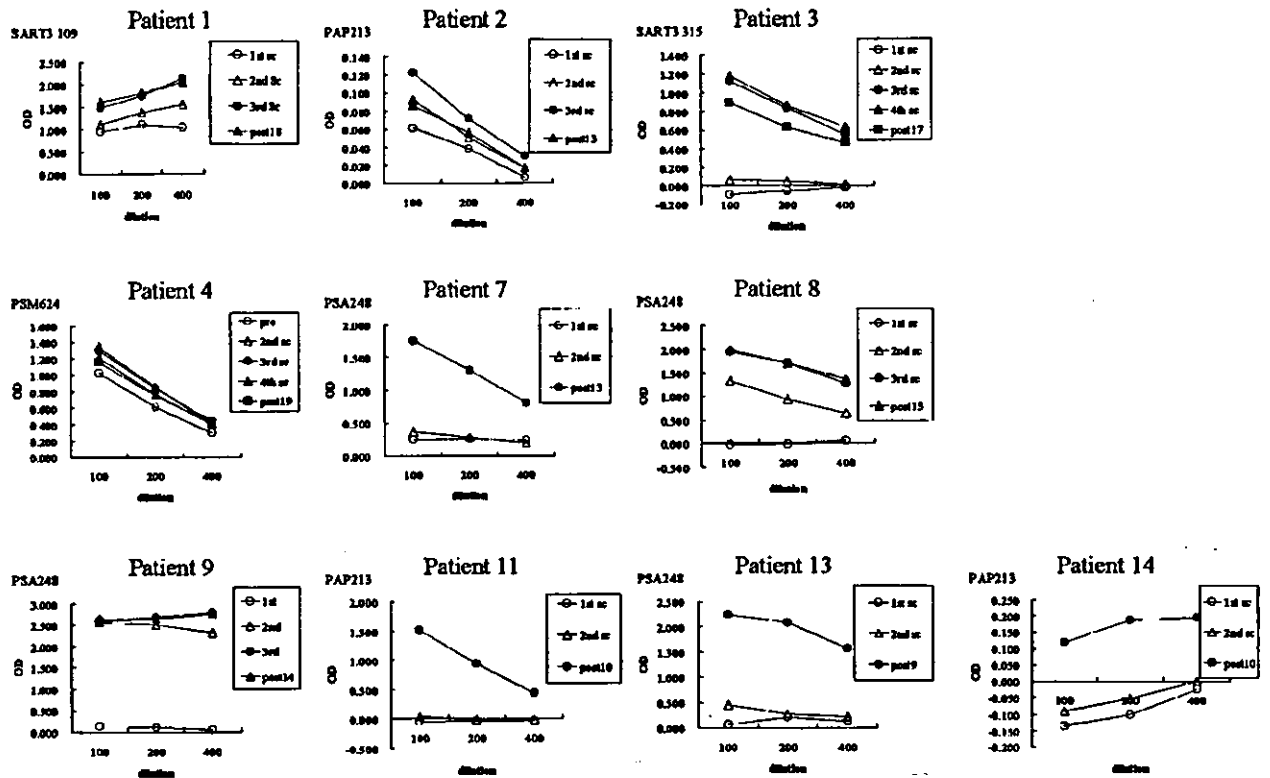


Fig. 2. Serial changes of IgG levels specific to the peptides administered. Pre- and post-vaccination sera were serially diluted and the levels of peptide-specific IgG were measured using ELISA, as described in Materials and Methods. Horizontal lines indicated OD and vertical lines indicate dilution of sera.

and a summary is presented in Table III. There are several patterns of immune responses. One pattern is the group in which both CTL and IgG responses were augmented (Patients #1, 4, 7, and 8). Only CTL response was augmented in Patients #10 and 12, while it was opposite in Patients #3, 9, and 11.

**Clinical Responses**

Among the 16 patients, two patients had rapid tumor progression before the combination therapy, and the remaining one patient withdrew his consent from combination therapy after a serum PSA level decrease of  $\geq 50\%$  with vaccination alone. The remaining 13 patients received the combination of vaccinations and a low dose of estramustine phosphate, and were evaluated for clinical responses. All 13 patients showed a serum PSA level decrease from the baseline after the combination therapy, and 6 of 13 (46%) patients showed a serum PSA level decrease of  $\geq 50\%$  with a median duration of 7.5 months (range, 3–13 months). However, none of these 13 patients had objective response to treatment.

QOL outcomes of 16 patients during this treatment were evaluated at pre-vaccination (16 patients), and 3rd

(16 patients), 6th (13 patients), and 12th vaccination (7 patients) using a Japanese version of questionnaire of the FACT-P. Figure 3 shows an average percentage of scales for each factor at the measuring point. QOL outcomes for all factors were not deteriorated during the treatment.

At the present time, five patients have died and all deaths were attributed to prostate cancer or metastases. The median survival rate has not been calculated with a median follow-up of 11 months ranging from 5 to 17 months. At 12 months, 61.5% of patients were still alive. Figure 4 shows the cancer related survival for 13 patients in this study comparing the survival of a previous study [9], in which 8 of 10 patients were treated by a combination of estramustine phosphate and vaccination without peptides derived from PAP, PSA, and PSMA. The survival in the present study demonstrated a greater trend of improvement than that in the previous study, but there was no statistical difference between the survival rates of the two groups.

**DISCUSSION**

The 16 peptides used in this study had the ability to induce HLA-A24-restricted and tumor-specific CTL in