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 *SalI* 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-A33$^-$ tumor cells, COS-7 cells, natural killer target cell line K562, or HLA-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

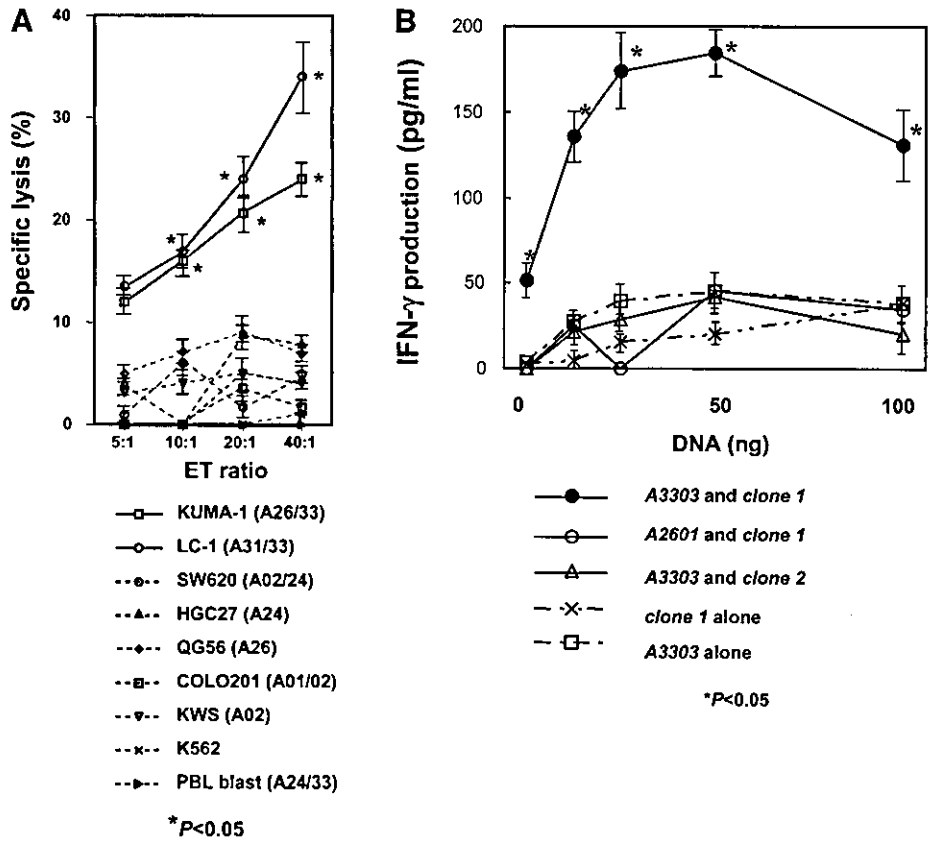

Fig. 1 Identification of the gene recognized by the HLA-A33–restricted 850B-CTL1. *A*. The cytotoxic activity of 850B-CTL1 against various target cells was tested by a 6-hour ^{51}Cr release assay at different E:T ratios. Values represent the means of triplicate determinations. Statistical analysis was 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.

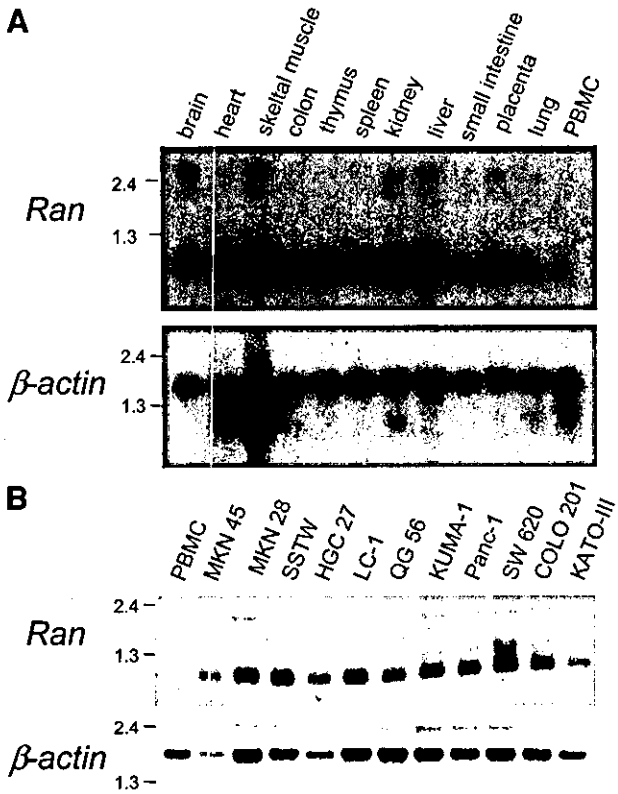

Fig. 2 Expression of *Ran* mRNA. *A*. The expression of *Ran* or control β-*actin* mRNA in normal tissues was examined by Northern blot analysis. *B*. The expression of *Ran* or control β-*actin* mRNA in a panel of cancer cell lines was examined by Northern blot analysis.

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

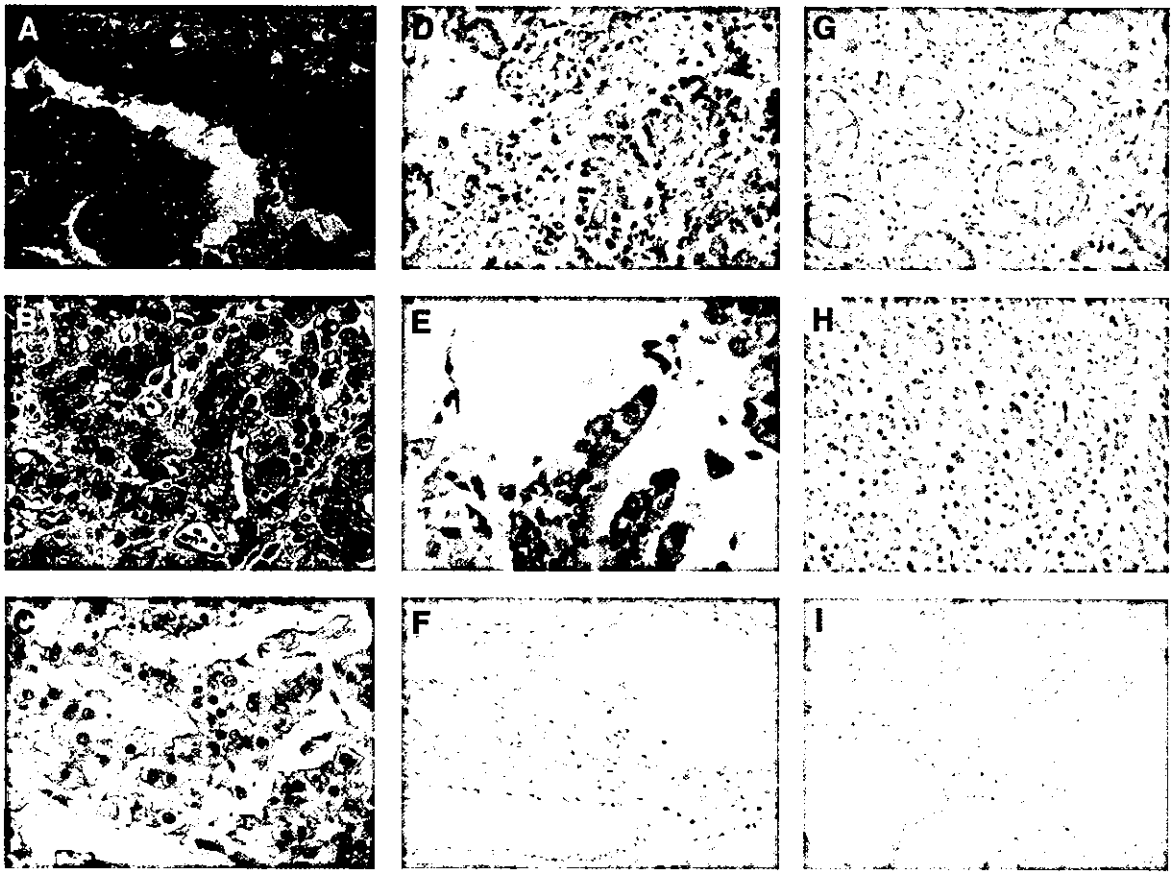

Fig. 3 Expression of Ran protein. Expression of Ran protein was examined by immunohistochemistry with anti-Ran mAb. Representative results of the histochemical stainings in cancer tissues (*A*, gastric; *B*, colon; *C*, pancreas; *D*, lung adenocarcinoma; *E*, lung squamous cell carcinoma) and normal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

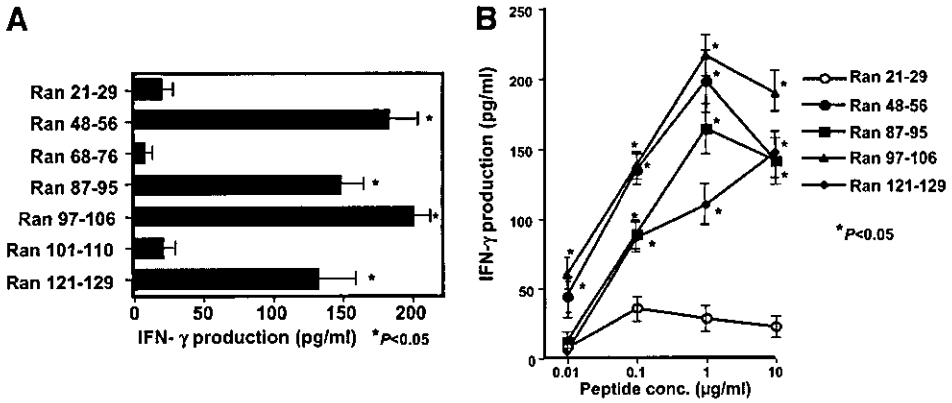

Fig. 4 Identification of CTL epitopes. *A*, determination of the antigenic peptides. Each of the seven peptides derived from Ran was loaded onto C1R-A33 cells at a concentration of 1 μg/mL. The 850B-CTL1 cells were cultured with the peptide-loaded C1R-A33 for 18 hours, and the culture supernatant was 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.

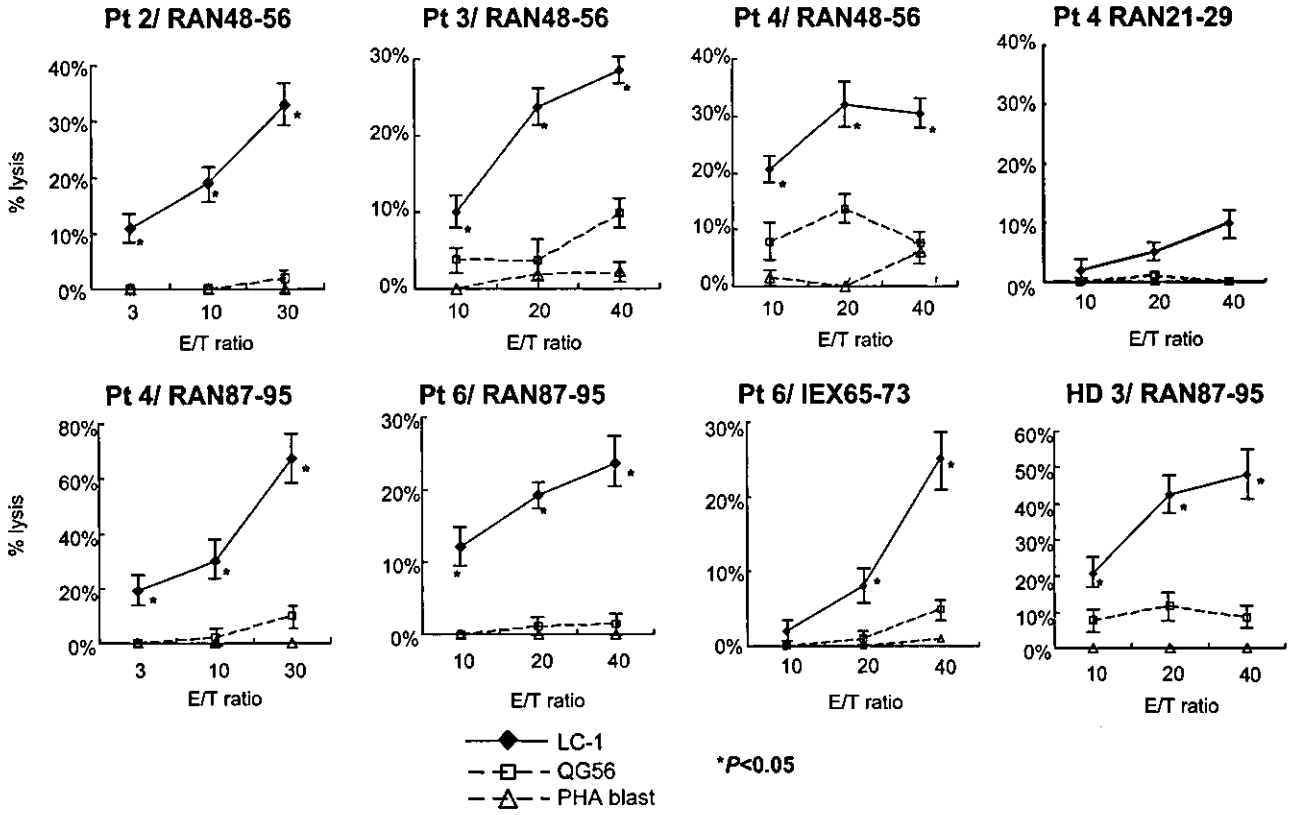

Fig. 5 Cytotoxity of the peptide-induced CTLs. Peptide-specific CTLs were induced by the Ran-derived peptides, Ran48-56 and Ran87-95, in the PBMCs from cancer patients. The cytotoxic activity of the peptide-induced CTLs against the LC-1 (HLA-A33$^+$) and QG56 (HLA-A33$^-$) tumor cells was measured by 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.

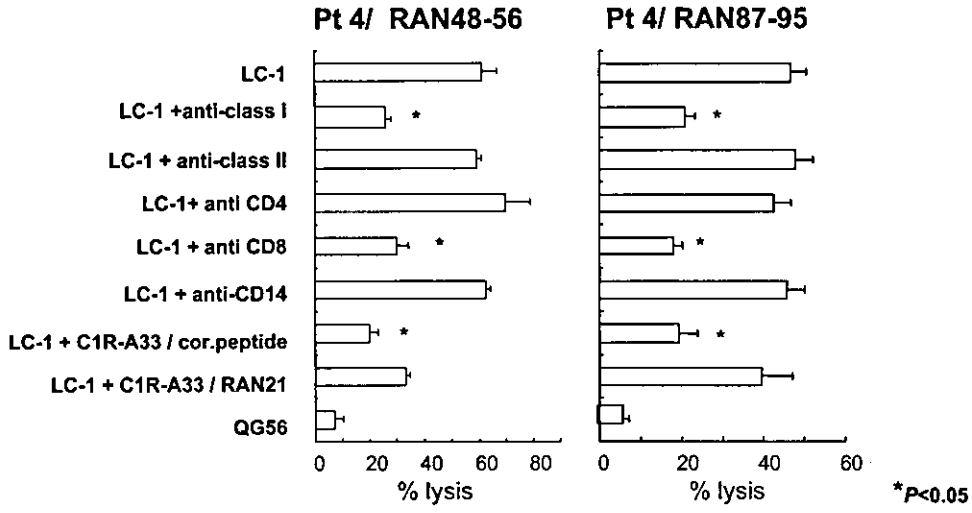

Fig. 6 Inhibition and competition assays. For the inhibition test, the peptide-stimulated PBMCs were tested for their restriction and for the peptide specificity of cytotoxicity against LC-1 (HLA-A33$^+$) by a standard 6-hour ^{51}Cr release assay in the presence or absence of 20 μg/mL anti-HLA class 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.

REFERENCES

1. Rosenberg SA. A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity 1999;10:281–7.

2. Rosenberg SA. Progress in human tumour immunology and immunotherapy. Nature (Lond.) 2001;411:380–4.

3. Ribas A, Butterfield LH, Glaspy JA, Economou JS. Current developments in cancer vaccines and cellular immunotherapy. J Clin Oncol 2003;21:2415–32.

4. Sato Y, Shomura H, Maeda Y, et al. Immunological evaluation of peptide vaccination for patients with gastric cancer based on pre-existing cellular response to peptide. Cancer Sci 2003;94:802–8.

5. Mine T, Gouhara R, Hida N, et al. Immunological evaluation of CTL precursor-oriented vaccines for advanced lung cancer patients. Cancer Sci 2003;94:548–56.

6. Tsuda N, Mochizuki K, Harada M, et al. Vaccination with predesignated or evidence-based peptides for patients with recurrent gynecologic cancers. J Immunother 2004;27:60–72.

7. Noguchi M, Kobayashi K, Suetsugu N, et al. Induction of cellular and humoral immune responses to tumor cells and peptides in HLA-A24–positive hormone-refractory prostate cancer patients by peptide vaccination. Prostate 2003;57:80–92.

8. Skipper JC, Hendrickson RC, Gulden PH, et al. An HLA-A2–restricted tyrosinase antigen on melanoma cells results from posttranslational modification and suggests a novel pathway for processing of membrane proteins. J Exp Med 1996;183:527–34.

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

10. Yang D, Nakao M, Shichijo S, et al. Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24–restricted cytotoxic T lymphocytes in cancer patients. Cancer Res 1999;59:4056–63.

11. Wang RF, Johnston SL, Southwood S, Sette A, Rosenberg SA. Recognition of an antigenic peptide derived from tyrosinase-related protein-2 by CTL in the context of HLA-A31 and -A33. J Immunol 1998;160:890–7.

12. Takedatsu H, Shichijo S, Katagiri K, et al. Identification of peptide vaccine candidates sharing among HLA-A3$^+$, -A11$^+$, -A31$^+$, -A33$^+$ cancer patients. Clin Cancer Res 2004;10:1112–20.

13. Sasada T, Takedatsu H, Azuma K, et al. IEX-1, a stress inducible anti-apoptotic gene, encodes CTL epitopes capable of inducing HLA-A33–restricted and tumor-reactive CTLs in gastric cancer patients. Cancer Res 2004;64:2882–8.

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

15. Aizawa, M. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. Oxford: Oxford University Press; 1992.

16. Weis K. Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. Cell 2003;112:441–51.

17. Kunzler M, Hurt E. Targeting of Ran: variation on a common theme? J Cell Sci 2001;114:3233–41.

18. Takiguchi M, Matsuda T, Tomiyama H, Miwa K. Analysis of three HLA-A*3303 binding peptide anchors using an HLA-A*3303 stabilization assay. Tissue Antigens 2000;55:296–302.

19. Takiguchi M, Matsuda T, Tomiyama H. Polarity of the P1 anchor residue determines peptide binding specificity between HLA-A*3101 and HLA-A*3303. Tissue Antigens 2000;56:501–6.

20. Hida N, Maeda Y, Katagiri K, Takasu H, Harada M, Itoh K. A simple culture protocol to detect peptide-specific cytotoxic T lymphocyte precursors in the circulation. Cancer Immunol Immunother 2002; 51:219–28.

21. Macara I, Brownawell, A, Ran Welch K. In: Hall A, editor. GTPases. Oxford: Oxford University Press; 2000. p. 198–221.

22. Lavia P, Mileo, AM, Giordano A, Paggi MG. Emerging roles of DNA tumor viruses in cell proliferation: new insights into genomic instability. Oncogene 2003;22:6508–16.

23. Li P, Yu X, Ge K, Melamed J, Roeder RG, Wang Z. Heterogeneous expression and functions of androgen receptor co-factors in primary prostate cancer. Am J Pathol 2002;161:1467–74.

24. Li SR, Gyselman VG, Dorudi S, Bustin SA. Elevated levels of RanBP7 mRNA in colorectal carcinoma are associated with increased proliferation and are similar to the transcription pattern of the proto-oncogene c-myc. Biochem Biophys Res Commun 2000;271:537–43.

25. Emberley ED, Gietz RD, Campbell JD, HayGlass KT, Murphy LC, Watson PH. RanBPM interacts with psoriasin in vitro and their expression correlates with specific clinical features in vivo in breast cancer. BMC Cancer 2002;2:28–34.

26. Gomi S, Nakao M, Niiya F, et al. A cyclophilin B gene encodes antigenic epitopes recognized by HLA-A24–restricted and tumor-specific CTLs. J Immunol 1999;163:4994–5004.

27. Harashima N, Tanaka K, Sasatomi T, et al. Recognition of the Lck tyrosine kinase as a tumor antigen by cytotoxic T lymphocytes of cancer patients with distant metastases. Eur J Immunol 2001;31:323–32.

28. Inoue Y, Takaue Y, Takei M, et al. Induction of tumor-specific cytotoxic T lymphocytes in prostate cancer using prostatic acid phosphatase derived HLA-A2402 binding peptide. J Urol 2001;166:1508–13.

29. Nakao M, Shichijo S, Imaizumi T, et al. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. J Immunol 2000;164:2565–74.

30. Yamada A, Kawano K, Koga M, Matsumoto T, Itoh K. Multidrug resistance-associated protein 3 is a tumor rejection antigen recognized by HLA-A2402–restricted cytotoxic T lymphocytes. Cancer Res 2001; 61:6459–66.

The Prostate 9999:1–12 (2004)

Immunological Evaluation of Individualized Peptide Vaccination With a Low Dose of Estramustine for HLA-A24$^+$ HRPC Patients

Masanori Noguchi,[1,3]* Kyogo Itoh,[2,3] Akihisa Yao,[2] Takashi Mine,[2] Akira Yamada,[2,3] Yayoi Obata,[2] Masatoshi Furuta,[2] Mamoru Harada,[2] Shigetaka Suekane,[1] and Kei Matsuoka[1]

[1]*Department of Urology, Kurume University, Kurume, Japan*
[2]*Department of Immunology, Kurume University, Kurume, Japan*
[3]*Kurume University School of Medicine, and Research Center of Innovative Cancer Therapy of The 21st Century Center of Excellence (COE) program for Medical Science, Kurume University, Kurume, Japan*

BACKGROUND. The safety, toxicity, and immunological response of individualized peptide vaccination or human leukocyte antigen (HLA)-A24^1 hormone refractory prostate cancer (HRPC) patients in combination with a low dose of estramustine were evaluated.
METHODS. Sixteen patients with HLA-A24^1 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-γ 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 administrated. 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 ≥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-A24^1 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

Grant sponsor: Japan Society for the Promotion of Science (Grants-in-Aid for Science Research (B) to MN); Grant number: 15591721.
*Correspondence to: Masanori Noguchi, MD, PhD, Department of Urology, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan. E-mail: noguchi@med.kurume-u.ac.jp
Received XXXXXX(01); Accepted XXXXXX
DOI 10.1002/pros.00000
Published online 00 Month 2004 in Wiley InterScience (www.interscience.wiley.com).

(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$^+$ 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)-γ-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$^+$ 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 radionuclide 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^3, hemoglobin greater than 10 g/dl, platelets greater than 100,000/mm^3, 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 ($SART1_{690-698}$, $SART2_{93-101}$, $SART2_{161-169}$, $SART2_{899-907}$, $SART3_{109-118}$, $SART3_{315-323}$, $Lck_{208-216}$, $Lck_{486-487}$, $Lck_{488-497}$, $ART1_{170-179}$, prostate acid phosphate $(PAP)_{213-221}$, $PSA_{152-160}$, $PSA_{248-257}$, prostate-specific membrane antigen $(PSMA)_{624-632}$, multidrug resistance-associated protein $(MRP)3_{503-511}$ and $MRP3_{1293-1302}$ 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 (1×10^5 cells/well) were incubated with 10 μM of each peptide in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark) in 200 μl 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-γ in response to C1R-A2402 cells preloaded with either a corresponding peptide or HIV peptide (RYLRQQLLGI) as a negative control. After incubation for 18 hr, the supernatants (100 μl) were collected, and the amounts of IFN-γ were measured using ELISA (limit of sensitivity: 10 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-γ (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 ≤net; A level of response (A): $P \leq 0.05$ and 50 ≤net; B: $P \leq 0.05$ and 25 ≤net <50; C: $0.05 < P \leq 0.1$ and 50 ≤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 μg/well) was blocked with Block Ace (Yukijirushi, Tokyo, Japan), washed, and 100 μl/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 μl 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 ml/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 μl 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 μg 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-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 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 mg/ml of each peptide was injected at 2- to 4-week intervals.

Estramustine phosphate was administered orally as a 140 mg capsule, 1 capsule twice daily, for a total daily dose of 280 mg as severe immunosuppression in the combination therapy was avoided by reducing estramustine phosphate to 280 from 560 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[a]			
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

[a]Performance status by EOCG 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

	Vaccine alone				Vaccine plus estramustine phosphate				
Toxicity[a]	G1	G2	G3	G4	G1	G2	G3	G4	Total
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

[a]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 theTherapy

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

10	SART2 93	57.3	228.5**	+	–	–	Combination	PR	13	14	Alive
	kk488	36.5	26.8	+	–	–					
	PSA152	49.3	0	+	+	–					
	PSA248	17	0	+	+	–					
11	SART3 109	625	250	+	+	–	Combination	–	–	11	Death
	kk486	375	58.5	-	+	–					
	PAP213	250	153.5	–	–	–					
	PSA248	250	38.8	–	++	–					
12	SART3 109	35.8	496	+	–	–	Vaccine	–	–	10	Death
	kk488	31.8	26.5	+	–	–					
	PAP213	28.8	622.3*	+	+	–					
	PSA248	18	243.5	+	+	–					
13	SART3 315	112.3	26.5	–	–	+(2)	Combination	–	–	5	Death
	PAP213	80	9.5	–	–	+(2)					
	PSA152	168.8	15.5	–	–	+(2)					
	PSA248	32.8	0	+	+	+(2)					
14	SART3 109	30.3	22.3	+	+	–	Combination	PR	9	11	Alive
	SART3 315	44.8	255	–	–	–					
	kk208	26.3	58.8	–	–	–					
	PAP213	14.3	324.3**	–	–	–					
15	SART2 161	0	n.a.	+	n.a.	–	Vaccine	–	–	5	Death
	SART3 109	25.3	n.a.	+	n.a.	–					
	SART3 315	15	n.a.	+	n.a.	–					
	PSA248	0	n.a.	+	n.a.	–					
16	SART3 109	2.8	0	+	n.a.	–	Combination	–	–	8	Alive
	kk488	30.5	19.8	–	n.a.	–					
	PAP213	33.8	32	+	n.a.	–					
	PSA248	23.8	9.8	+n.a.	–						

n.a., not available.

[a]The CTL precursor response to peptide was performed by qusdriduplicate assay. Means of specific IFN-γ production (pg/ml) of pre- and post(6th)-caccination PBMCs were calculated by subtracting the response to the HIV-derived irrelent peptide, and compared using Student's *t*-test. Increased IFN-γ production.

[b]–, absent; +, present, low titer; ++, present, high titer.

[c]Combination, vaccine plus estrarrustine phosphate; vaccine, vaccine only.

[d]PR, partial response.

*$P \leq 0.05$.

**$P \leq 0.10$.

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 $PSA_{248-257}$ (13/16), followed by $PAP_{213-221}$ (11/16), $SART3_{109-118}$ (11/16), $SART3_{315-323}$ (6/16), $PSA_{152-160}$ (5/16), $Lck_{488-497}$ (5/16), $SART1_{690-698}$ (3/16), $SART2_{93-101}$ (3/16), and $Lck_{208-216}$ (3/16). $PSMA_{624-628}$ and $SART2_{161-169}$ 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 administred to each patient. DTH response was observed in 4 of 16 patients,

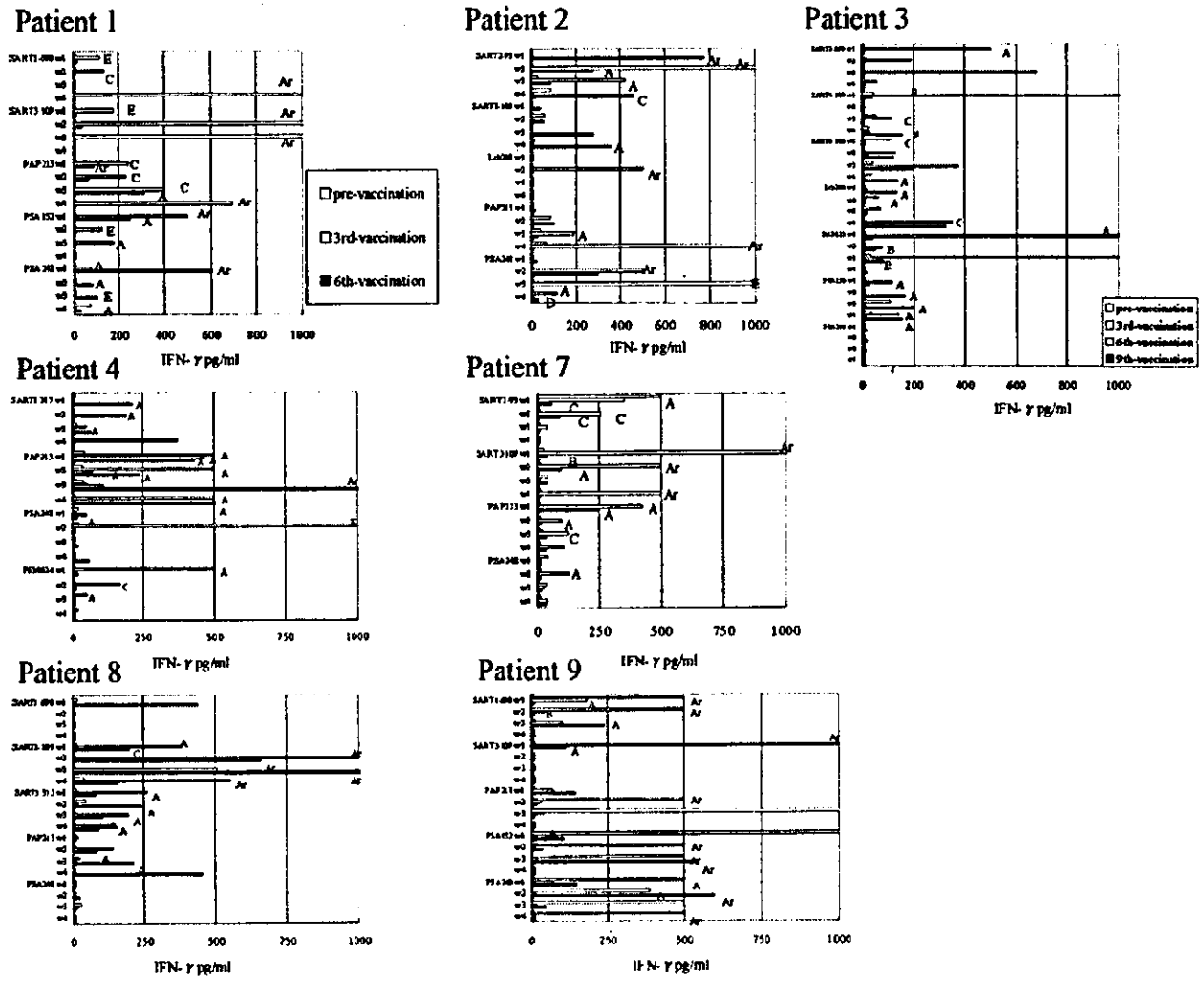

Fig. 1. [Q3] Representative results of peptide-specific CTL precursors in PBMCs of pre- and post-vaccinations. Four sets of columns in each peptide indicate the results of four well cultures and the value of each well was evaluated based on the following criteria. Armed response (Ar): $P \leq 0.01$ and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

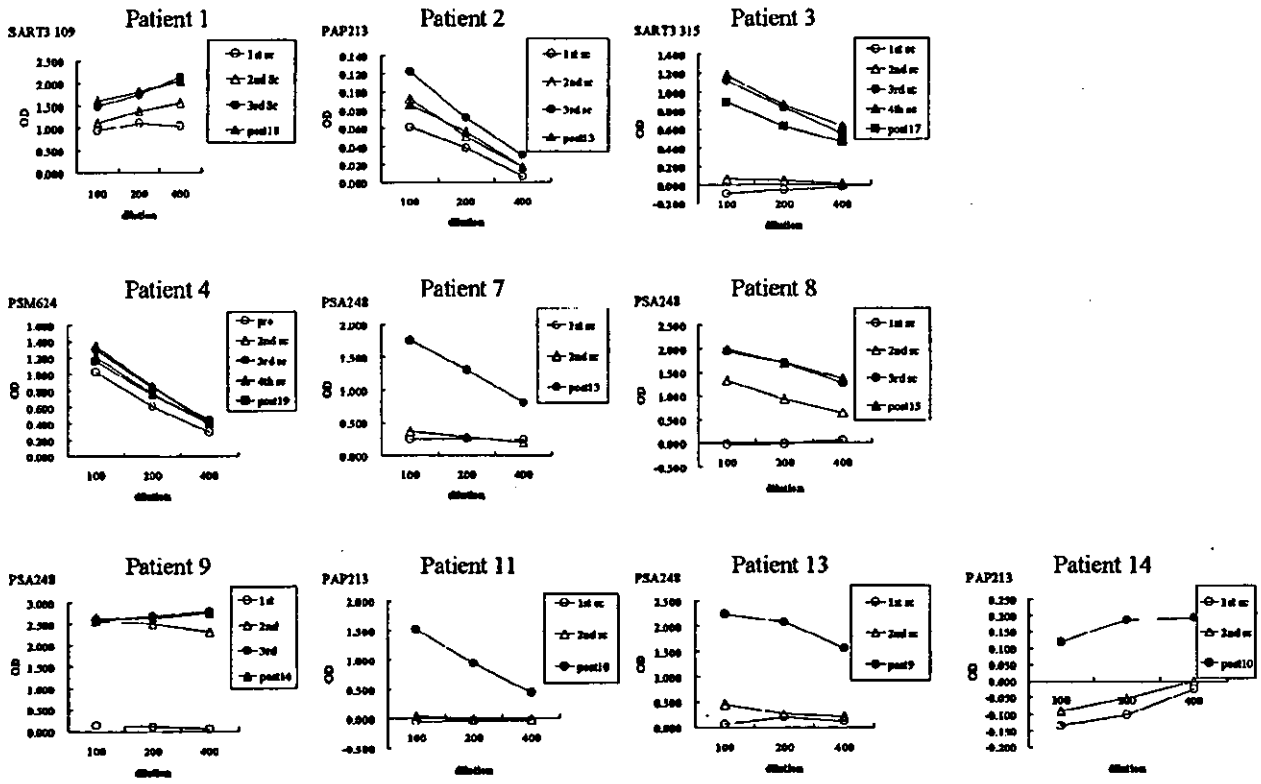

Fig. 2. Serial changes of IgG levels specific to the peptides administrated. Pre- and post-vaccination sera were serially diluted and the levels of peptide-specific IgG were measured using ELISA, as described in Materials and Methods. Horizontal lines indicated OD and vertical lines indicate dilution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

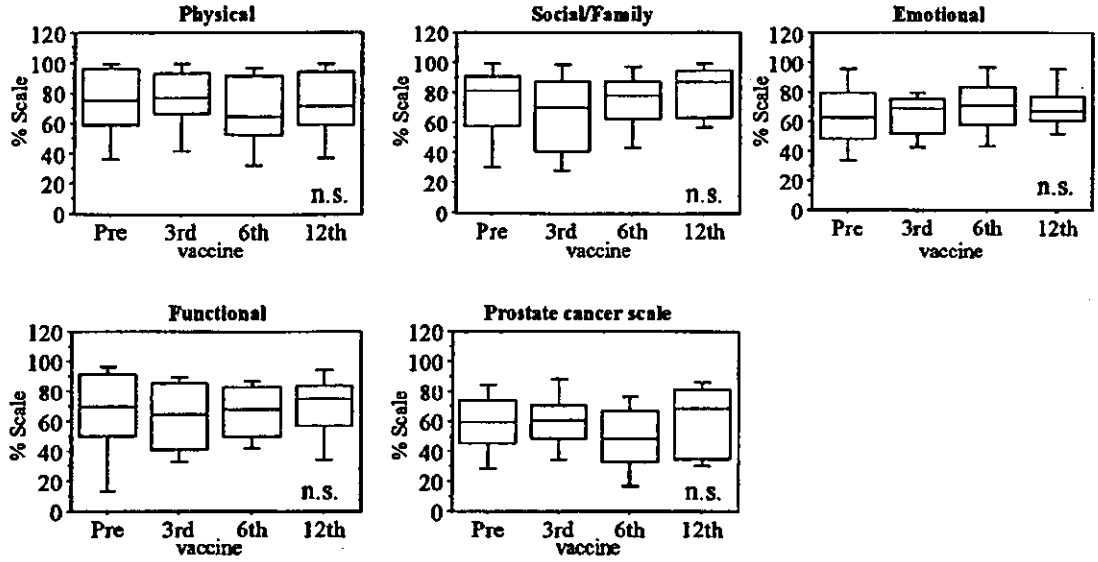

Fig. 3. Evaluation of % QOL scales during vaccination. QOL outcomes were separately assessed by the percentage of each scale. ns: not significant.

cancer patients. In the present study, 6 peptides including $PSA_{248-257}$, $PSA_{152-160}$, $PAP_{213-221}$, $PSMA_{624-632}$, $MRP3_{503-511}$, and $MRP3_{1293-1302}$ were added to the previously used 14 candidates for HLA-A24$^+$ patients with HRPC [9]. The most selected peptide in the present study was $PSA_{248-257}$, followed by $PAP_{213-221}$, $SART3_{109-118}$ and $PSA_{152-160}$, while $SART2_{93-101}$, $ART1_{188-196}$, $SART2_{899-907}$, $SART3_{109-118}$, and $Lck_{208-216}$ were selected frequently in the previous study [9]. For specific immunotherapy, it is necessary to determine antigens and epitope peptides that can be recognized by the immune response. We recently reported that both the $PSA_{248-257}$ and $PSA_{152-160}$ peptides are immunogenic in HLA-A24 positive prostate cancer patients, and that the peptide-stimulated

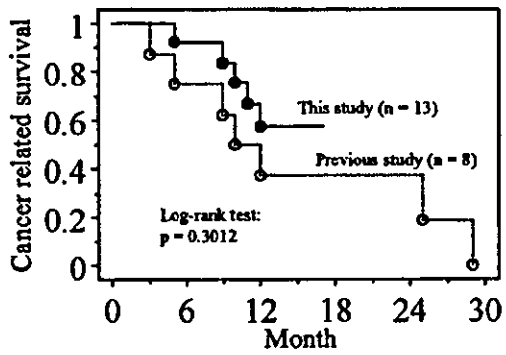

Fig. 4. Prostate cancer related survival. There were five observed deaths in the present study group with a median follow-up of 11 months vs. 7 in the previous study group (Noguchi et al., 2003a) with a median follow-up of 13 months. The median survival rate in the present study has not been calculated.

PBMCs of them can show cytotoxicity against prostate cancer cells in an HLA-A24-restricted manner [13]. Inoue et al. revealed that the $PAP_{213-221}$ peptide can induce peptide-specific CTLs from HLA-A2402 positive prostate cancer patients [12]. PSMA is an integral membrane protein and its expression can be detected on prostate carcinoma, even on HRPC [26]. The $PSMA_{624-632}$ peptide was reported to be immunogenic in HLA-A24 positive prostate cancer patients [14]. Also, several antigens, which are not prostate tissue-specific but are highly expressed in prostate cancer, have been identified. Expression of SART2, SART3, and Lck ($p56^{lck}$) proteins was observed in prostate cancer cell line and tissues [9]. Interestingly, in this study, IgGs specific to these CTL epitope peptides were detected in 7 of 14 patients after six vaccinations and 10 of 12 patients during the treatment. These findings suggested these peptides could be recognized by both T-cell and B-cell immunity.

To activate peptide-specific CTLs with peptide vaccine, peptide-pulsed Langerhans cells must come into contact with peptide-specific CTL precursor cells in regional lymph nodes within 2 days [27]. Therefore, determining the frequency of peptide-specific CTL precursors in PBMCs will be important to achieve antitumor immunity. In addition, multiple peptides may be needed to prevent prostate cancer cells from immune escaping. The present approach in immunotherapy for HRPC patients used a new strategy of a pre-vaccination measurement of both humoral and cellular responses to peptides, followed by administration of up to four peptides that had been reactive for pre-vaccination measurement among 16 vaccine candidates (individualized

peptide vaccination). Previous results from a phase I study demonstrated that individualized peptide vaccination was feasible, safe, and immunologically active, but the clinical response has been largely limited [9]. In the present study, all of 16 patients except for one patient also showed an increase of PSA levels during vaccination therapy alone, while augmentation of peptide-specific CTL precursors or peptide-specific IgG was observed in the majority of the patients treated. One of the possible explanations for this discrepancy between immunological and clinical responses is HLA class I antigen down-regulation in prostate cancer. HLA class I antigen down-regulation in prostate cancer may have a negative impact on the outcome of T-cell-based immunotherapy because tumor-specific CTLs failed to show reacting to class I-loss variants. On the contrary, the majority of patients in this study had initially failed to respond to treatments with estramustine phosphate for prostate cancer, whose cells were mostly resistant to estramustine phosphate. Regardless of these factors, the results of the present phase I/II study suggested a benefit by combining individualized peptide vaccination with a low dose of estramustine phosphate in patients with HRPC. The response rate (46%) defined as a serum PSA level decrease of $\geq$50% with a median duration of 7.5 months in the present study is comparable with the response rate in recently reported chemotherapy trials with combinations such as estramustine and paclitaxel (53%) [28], estramustine and docetaxel (62%) [29], and the three-drug combination of estramustine, peclitaxel, and carboplatin (67%) [30]. In other reports, estramustine with paclitaxel and etoposide can prolong the mean survival time (23.4 months), whereas, the regime seems to be accompanied with severe adverse effects [1]. Our results suggested that additive anti-tumor effects could be achieved by the combination of T-cell-based immunotherapy and cytotoxic agents with minimum immunosuppression. In the present study, PSA responses were observed in patients who experienced disease progression prior to estramustine phosphate or peptide vaccination alone, supporting the hypothesis that prostate cancer cells do not have resistance to both vaccination and estramustine phosphate. Cancer cells are extremely robust for their own growth and survival against various perturbations. The highly effective administration of multiple drug regimes can be accomplished only with a system-level analysis of the dynamics of gene regulatory circuits [31]. Further studies on the systems therapy are needed.

The toxicity of the combination regimen reported here was tolerable and this treatment was considered acceptable in the treatment of the vast majority of metastatic HRPC. The most common toxicities were dermatologic reactions at the injection site of the vaccination. There was no hematologic toxicity nor neuropathy reported in estramustine-based or taxane-based chemotherapy regimens for patients with HRPC, and they were dose-limiting toxicities [28–30]. The common toxicities of estramustine treatment include nausea, vomiting, peripheral edema, and vascular events [28]. The incidence of those toxicities in the present study was low because of a low-dose setting of estramustine phosphate. We previously reported that increased humoral response but not cellular response well correlated with overall survival of the vaccinated patients [20]. This issue seemed to be confirmed in this study, although this small scale of the clinical trial alone could not provide any definitive conclusion to a laboratory marker to predict overall survival.

For patients with advanced disease who have reduced life expectancy and no immediate hope for a cure, relief of physical symptoms, and maintenance of function become primary objectives of medical intervention [32]. Systematic symptom assessment may help to clarify a treatment's toxicity, potential palliative benefit, or need to make a change in the patient's clinical management. In the present study, the % QOL scales using FACT-P were stable and well during the treatment, and all patients were treated as out patients.

Based on these findings, we are undertaking larger phase II studies of this regimen.

REFERENCES

1. Millikan R, Thall PF, Lee SJ, Jones D, Cannon MW, Kuebler JP, Wade J III, Logothetis CJ. Randomized, multicenter, Phase II trial of two multicomponent regimens in androgen-independent prostate cancer. J Clin Oncol 2003;21:878–883.
2. Van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 1991;254:1643–1647.
3. Kawakami Y, Eliyahu S, Sakaguchi K, Robbins PF, Rivoltini L, Yannelli JR, Appella E, Rosenberg SA. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. J Exp Med 1994;180:347–352.
4. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Dudley ME, Schwarz SL, Spiess PJ, Wunderich JR, Parkhust MA, Kawakami Y, Seipp CA, Einhorn JH, White DE. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998;4:321–327.
5. Marchand M, van Baren N, Weynants P, Brichard V, Dreéno B, Tessier MH, Rankin E, Parmiani G, Arienti F, Humblet Y, Bourlond A, Vamwijck R, Liénard D, Beauduin M, Dietrich PY, Russo V, Kerger J, Masucci G, Jäger E, De Greve J, Atzpodien J, Brasseur F, Coulie PG, van der Bruggen P, Boon T. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene *MAGE*-3 and presented by HLA-A1. Int J Cancer 1999;80:219–230.
6. Hsu FJ, Benike C, Fagononi F, Liles TM, Czerkinski D, Taidi B, Engleman EG, Levy R. Vaccination of patients with B cell

lymphoma using autologous antigen-pulsed dendritic cells. Nat Med 1996;2:52–58.

7. Nestle FO, Alijagic S, Gilliet M, Yuansheng S, Grabbe S, Dummer R, Burg G, Schandendorf D. Vaccintion of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 1998;4:328–332.
8. Tjoa BA, Simmons SJ, Elgamal A, Rogers M, Ragde H, Kenny GM, Troychak MJ, Boynton AL, Murphy GP. Follow-up evaluation of a phase II prostate cancer vaccine trial. Prostate 1999;40:125–129.
9. Noguchi M, Kobayashi K, Suetsugu N, Tomiyasu K, Suekane S, Yamada A, Itoh K, Noda S. Induction of cellular and humoral immune responses to tumor cells and peptides in HLA-A24 positive hormone-refractory prostate cancer patients by peptide vaccination. Prostate 2003;87:80–92.
10. Noguchi M, Itoh K, Suekane S, Yao A, Suetsugu N, Katagiri K, Yamada A, Yamana H, Noda S. Phase I trial of patient-oriented vaccination in HLA-A2 positive patients with metastatic hormone refractory prostate cancer. Cancer Sci 2004;95:77–84.
11. Noguchi M, Itoh K, Suekane S, Morinaga A, Sukehiro A, Suetsugu N, Katagiri K, Yamada A, Noda S. Immunological monitoring during combination of patient-oriented peptide vaccination and estramustine phosphate in patients with metastatic hormone refractory prostate cancer. Prostate (in press[Q2])
12. Inoue Y, Takaue Y, Takei M, Kato K, Kanai S, Harada Y, Tobisu K, Noguchi M, Kakizoe T, Itoh K, Wakasugi H. Induction of tumor specific cytotoxic Tlymphocytes in prostate cancer using prostatic acid phosphatase derived HLA-A2402 binding peptide. J Urol 2001;166:1508–1513.
13. Harada M, Kobayashi K, Matsueda S, Nakagawa M, Noguchi M, Itoh K. Prostate-specific antigen-derived epitopes capable of inducing cellular and humoral responses in HLA-A24$^+$ prostate cancer patients. Prostate 2003;57:152–159.
14. Kobayashi K, Noguchi M, Itoh K, Harada M. Identification of a prostate-specific membrane antigen-derived peptide capable of eliciting both cellular and humoral immune responses in HLA-A24$^+$ prostate cancer patients. Cancer Sci 2003;94:622–627.
15. Harada M, Noguchi M, Itoh K. Target molecules in specific immunotherapy against prostate cancer. Int J Clin Oncol 2003;8: 193–199.
16. Yang D, Nakao M, Shichijo S, Sasatomi T, Takasu H, Matsumoto H, Mori K, Hayashi A, Yamana H, Shirouzu K, Itoh K. Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24-restructed cytotoxic T lymphocytes in cancer patients. Cancer Res 1999;59: 4056–4063.
17. Gomi S, Nakao M, Niiya F, Imamura Y, Kawano K, Nishizaka S, Hayashi A, Sobao Y, Oizumi K, Itoh K. A cyclophilin B gene encodes antigenic epitopes recognized by HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes. J Immunol 1999; 163:4994–5004.
18. Harashima N, Tanaka K, Sasatomi T, Shimizu K, Miyagi Y, Yamada A, Tamura M, Yamana H, Itoh K, Shichijo S. Recognition of the Lck tyrosine kinase as a tumor antigen by cytotoxic T lymphocytes of cancer patients with distant metastases. Eur J Immunol 2000;31:323–332.
19. Kawano K, Gomi S, Tanaka K, Tsuda N, Kamura T, Itoh K, Yamada A. Identification of a new endoplasmic reticulum-resident protein recognized by HLA-A-24 restricted tumor infiltrating lymphocytes of lung cancer. Cancer Res 2000;60: 3550–3558.
20. Mine T, Sato Y, Noguchi M, Sasatomi T, Gouhara R, Tsuda N, Tanaka S, Shomura H, Katagiri K, Rikimaru T, Shichizo S, Kamura T, Hashimoto T, Shirouzu K, Yamada A, Todo S, Itoh K, Yamana H. Humoral responses to peptides correlate with overall survival in advanced cancer patients vaccinated with peptides based on pre-existing peptide-specific cellular responses. Clin Cancer Res 2004;10:929–937.
21. Miyagi Y, Imai N, Sasatomi T, Yamada A, Mine T, Katagiri K, Nakagawa M, Muto A, Okouchi S, Isomoto H, Shirouzu K, Yamana H, Itoh K. Induction of cellular immune response to tumor cells and peptides in colorectal cancer patients by vaccination with SART3 peptides. Clin Cancer Res 2001;7: 3950–3962.
22. Mine T, Gouhara R, Hida N, Imai N, Azuma K, Rikimaru T, Katagiri K, Nishikori M, Sukehiro A, Nakagawa M, Yamada A, Aizawa H, Shirouzu K, Itoh K, Yamana H. Immunological evaluation of CTL precursor-oriented vaccines for advanced lung cancer patients. Cancer Sci 2003;94:548–556.
23. Noguchi M, Kikuchi H, Ishibashi M, Noda S. Percentage of the positive area of bone metastasis is an independent predictor of the disease death in advanced prostate cancer. Br J Cancer 2003; 88:195–201.
24. Esper PMoF, Chodak G., Sinner M, Cella D, Pienta KJ. Measuring quality of life in men with prostate cancer using the functional assessment of cancer therapy-prostate instrument. Urology 1997; 50:920–928.
25. Hinotsu A, Niimi M, Akaza H, Miyanaga N, Takeshima H, Eremenco S, Cella D. Development of Japanese version of QOL questionnaire for bladder and prostate cancer patients using FACT-B and P. Jpn J Cancer Chemother 1999;26:657–666.
26. Israeli RS, Powell CT, Fair WR, Heston WD. Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. Cancer Res 1993;53:227–230.
27. Janeway CA, Travers P, Walport M. Immunobiology. 4th edn. New York: Current Biology Publications & Garland Publishing. 1999. pp 391–393.
28. Hudes GR, Nathan F, Khater C, Haas N, Cornfield M, Giantonio B, Greenberg R, Gomella L, Litwin S, Ross E, Roethke S, McAleer C. Phase II trial of 96-hour paclitaxel plus oral estramustine phosphate in metastatic hormone-refractory prostate cancer. J Clin Oncol 1997;15:3156–3163.
29. Ellerhorst JA, Tu SM, Amato RJ, Finn L, Millikan RE, Pagliaro LC, Jackson A, Logothetis CJ. Phase II trial of alternating weekly chemohormonal therapy for patients with androgen-independent prostate cancer. Clin Cancer Res 1997;3:2371–2376.
30. Kelly WK, Curley T, Slovin S, Heller G, McCaffrey J, Bajorin D, Ciolino A, Regan K, Schwartz M, Kantoff P, George D, Oh W, Smith M., Kaufman D, Small EJ, Schwartz L, Kantoff P, George D, Oh W, Smith M, Kaufman D, Small EJ, Schwartz L, Larson S, Tong W, Scher H. Paclitaxel, estramustine phosphate, and carboplatin in patients with advanced prostate cancer. J Clin Oncol 2001;19:44–53.
31. Kitano H. Computational systems biology. Nature 2002;420: 206–210.
32. Coons SJ, Kaplan RM. Assessing health-related quality-of-life: Application to drug therapy. Clin Ther 1992;14:850–858.

Q1: Please provide the history dates.

Q2: Au: Please update.

Q3: Au: Please provide the subparts a, b and c in the artworks.