TABLE 1
Patient characteristics

Characteristics	No. of patients
Gender	
Male	8
Female	2
HLA typing	
A-2	4
A-24	5
A-2 and A-24	1
Primary organ	
Bladder	7
Upper urinary tract	2
Both	1
Surgical management	
TURBT	7
Nephroureterectomy	2
Radical cystectomy	1
Main target tumour	
Lymph node	5
Bladder	3
Bone	2
Previous treatment	
Chemotherapy	5
Chemotherapy and radiation therapy	5
Performance status*	
0	5
1	5

HLA, human leucocyte antigen; TURBT, Transurethral resection of bladder tumour.
**Performance status by Eastern Cooperative Oncology Group score.*

produce interferon-γ (IFN-γ) in response to CIR-A2402 (kindly provided by Dr M. Takiguchi, Kumamoto University, Japan) or T2 cells that were pre-loaded with either a corresponding peptide or HIV peptides (RYLRQQLLGI for HLA-A24 and LLFGYPVYV for HLA-A2) as a negative control. The level of IFN-γ was determined by ELISA (limit of sensitivity: 10 pg/mL). All assays were performed in quadruplicate. A two-tailed Student's t test was employed for the statistical analyses. A well was considered positive when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($P < 0.05$) than that in response to an HIV peptide, and when the mean amount of IFN-γ production in response to a corresponding peptide was >50 ng/mL compared with that in response to an HIV peptide. The increment of CTL activity was judged as positive if the post-vaccination sample, but not the pre-vaccination sample, showed CTL activity. It was also judged as positive if the level of IFN-γ produced by the post-vaccination (12th) sample was twice as high as that produced by the pre-vaccination sample. Our previous study showed that both increased IgG and a CTL response at least twice that of the vaccinated peptides correlated well with overall survival in patients with castration-resistant prostate cancer [22].

The levels of anti-peptide IgG were measured using the Luminex™ system, as previously reported [23]. In brief, plasma was incubated with 25 μL peptide-coupled colour-coded beads for 2 h at room temperature on a plate shaker. After incubation, the mixture was washed with a vacuum manifold apparatus and incubated with 100 μL biotinylated goat anti-human IgG (chain-specific) for 1 h at room temperature. The plate was then washed, 100 μL of streptavidin-phycoerythrin was added to the wells, and the mixture was incubated for 30 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 μL Tween-PBS into each well. Fifty microlitres of sample was detected using the Luminex™ system. The sample was judged to be positive if the IgG level of the post-vaccination (12th) plasma was twice as high as that of the pre-vaccination plasma. This definition is the same as the CTL response according to our previous results [22].

Standard indirect immunoperoxidase procedures (ENVISION Kit; DakoCytomation California, Carpinteria, CA, USA) in combination with monoclonal antibodies were used for the detection of infiltrating lymphoid cells (CD45RA and CD45RA, 1:50; Dako, Glostrup, Denmark) [24]. Cells with known positive results were used as positive controls. The primary antibody was omitted for negative controls.

Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. The clinical response was evaluated based on clinical observations and radiological findings. All known sites of disease were evaluated every 6 weeks by CT scan or MRI examination before and after each cycle. During treatment, blood counts and serum chemistries were performed weekly. Patients were assigned a response category according to the Response Evaluation Criteria in Solid Tumors (RECIST).

Student's t test was employed for evaluation of immunological assays. Progression-free survival time, overall survival time and response duration were calculated from the first day of peptide vaccination until the date of disease progression or death. The time-to-event endpoint was derived by the Kaplan–Meier method. All patients entering the trial were included in the survival determinations.

RESULTS

Between July 2007 and April 2009, 10 patients were treated with peptide vaccination at our institutions. Data were collected until December 2009. One patient did not meet the protocol entry criteria because cisplatin-based chemotherapy had not been received before the peptide vaccination. Median age was 71 years (range 44–77 years). Median follow-up time was 8.9 months (mean 12.0 months, range 2.5–29.3 months). Seven patients had bladder UC, two patients had upper urinary tract UC and one patient had bladder and upper urinary tract UC. Seven patients had metastatic disease, of whom five had lymph node metastasis and two had bone metastasis; three patients had locally advanced UC without distant metastasis after MVAC chemotherapy. The clinical characteristics of all entry patients are listed in Table 1.

For the selection of peptides for the first to 12th vaccinations (the first cycle), pre-

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TABLE 2 Immune responses and clinical outcomes

Patient no. Clinical stage	Peptide	No. of vaccinations	Cellular response* Pre-	After 12th	Anti-peptide IgG† Pre-	After 12th	Clinical response	PFS (months)	OS (months)	Prognosis
1 T4N0M1	PAP-213	10	–	NA	1753	NA	PD	1	3	Dead
	PSA-248		–	NA	110	NA				
	EZH2-735		–	NA	51	NA				
	PTHrP-102		–	NA	149	NA				
2 T3bN0M0	SART3-109	24	–	–	193	238	PR	22	28	Alive
	Lck-486		–	–	45	43				
	MRP3-1293		–	–	128	180				
	PAP-213		–	1923	167	23 959				
3 TisN2M1	SART3-109	12	–	–	48	13 261	PD	3	5	Dead
	Lck-486		155	–	53	156				
	MRP3-1293		–	–	228	2 144				
	PAP-213		–	–	353	25 892				
4 T3bN2M0	SART3-109	25	158	137	341	26 423	SD	22	29	Alive
	Lck-488		–	327	195	769				
	PAP-213		–	207	344	22 943				
	SART2-92		68	162	214	221				
5 T3bN1M0	MAP-432	12	–	113	37	128	CR	20	20	Dead
	Lck-422		–	216	32	25				
	WHSC2-103		57	2558	15	19				
	UBE2V-85		–	2684	20	26				
6 T3bN1M0	SART3-309	12	117	198	66	61	PD	3	4	Dead
	CypB-129		–	–	99	90				
	UBE2V-43		–	–	174	303				
	HNRPL-501		–	548	55	41				
7 T4aN2M1	SART3-109	12	–	–	62	25 796	PD	3	9	Dead
	Lck-486		–	–	31	42				
	Lck-488		–	–	89	131				
	UBE2V-43		–	6212	72	272				
8 T4N2M0	Lck-422	23	–	251	47	4 315	SD	3	9	Dead
	UBE2V-43		–	–	61	12 296				
	WHSC2-141		–	–	27	44				
	HNRPL-140		–	209	30	257				
9 T3N1M0	Lck-422	12	–	–	37	1 395	PD	4	5	Dead
	UBE2V-43		–	3252	129	11 845				
	HNRPL-140		–	–	33	2 231				
10 T4N0M0	Lck-208	16	–	193	216	232	PD	3	9	Alive
	MRP3-1293		–	1712	368	438				
	PAP-213		514	551	357	3 161				
	PSA-248		–	–	711	5 588				

*CR, complete response; NA, not available; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease. *Values indicate interferon-γ (IFN-γ) production of peripheral blood mononuclear cells reactive to the corresponding peptide (pg/mL). A two-tailed Student's t test was employed for the statistical analysis. A well was considered positive when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($P < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFN-γ production in response to a corresponding peptide was >50 ng/mL, compared with that to an HIV peptide. Increment of cytotoxic T lymphocyte activity was judged as positive if the post-vaccination samples, but not the pre-vaccination samples, showed the cytotoxic T lymphocyte activity. It was also judged as positive if the level of IFN-γ produced by the post-vaccination sample was more than twice as high as that produced by the pre-vaccination sample. The values showing the increment are underlined. †Plasma levels of peptide-specific IgG were measured using the Luminex™ system. Values indicated fluorescence intensity units of IgG antibodies reactive to the corresponding peptide. The sample was judged positive if the IgG level of the post-vaccination (12th) plasma was twice as high as that of the pre-vaccination plasma. The values showing positive response are underlined.*

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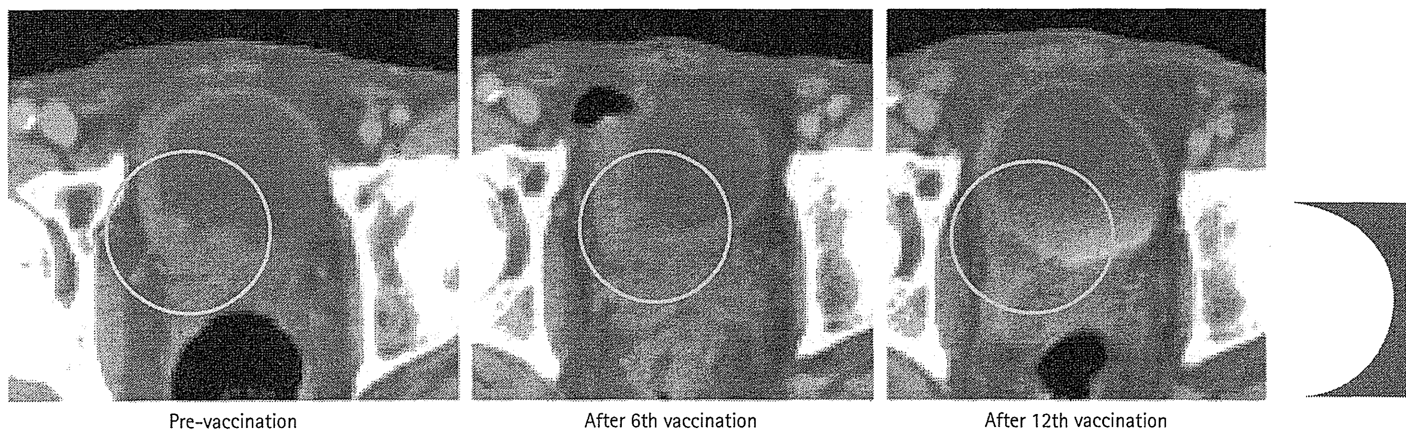
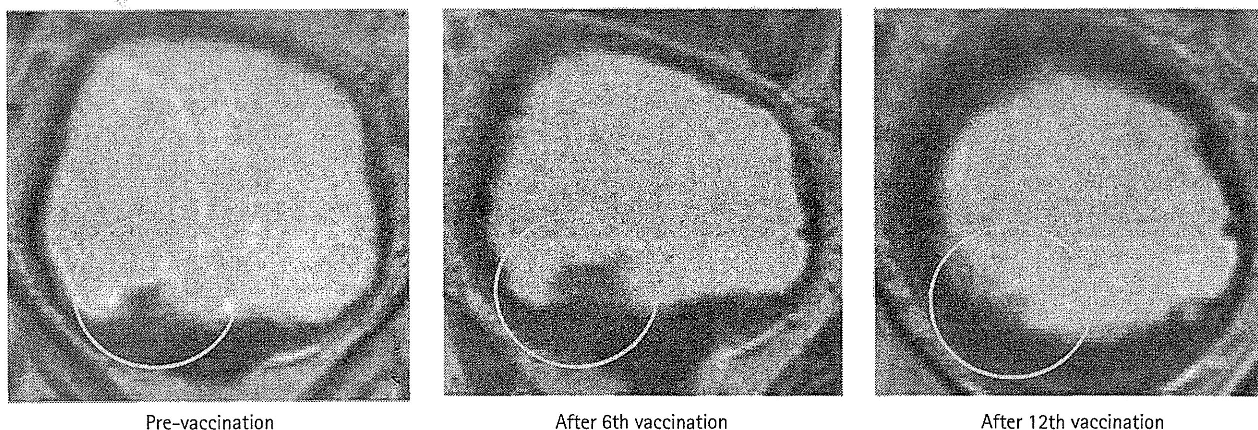
FIG. 1. The kinetic CT images of the tumour lesion of a patient with complete remission (A) and a patient with partial remission (B). The yellow circle indicates the tumour region. Left: pre-vaccination; middle: after the sixth vaccination; right: after the 12th vaccination. Cystoscopy findings of the patient with complete remission after the 12th vaccination showed no visible tumours with negative urinary cytology and post-inflammatory lesions.

(A) Complete remission



Pre-vaccination | After 6th vaccination | After 12th vaccination

(B) Partial remission



Pre-vaccination | After 6th vaccination | After 12th vaccination

vaccination plasma was used to investigate the reactivity to each of the 14 or 16 peptides in the HLA-A24$^+$ ($n = 5$) or HLA-A2$^+$ patients ($n = 4$), respectively, followed by selection of the three or four peptides with higher levels of IgG reactivity to each of the peptides in order. For the one patient who was HLA-A24$^+$ and HLA-A2$^+$, all 30 peptides were used for the selection of peptides followed by selection of three peptides from the 14 peptides used for HLA-A24$^+$ patients and the remaining one peptide from the 16 peptides used for HLA-A2$^+$ patients; the peptides chosen had the higher levels of IgG reactivity. A summary of the administered peptides is shown in Table 2. For the second cycle (13th to 24th), the four peptides with highest reactivities were similarly chosen for administration on the basis of the results of screening both PBMCs and plasma. Eight patients received twelve vaccinations and two patients received twenty-four vaccinations without other chemotherapy treatment.

Representative non-haematological toxicity consisted of dermatological skin reactions including redness and heat at the vaccination site in all patients with grade 1 or 2 toxicity. There were no haematological toxicities or therapy-related deaths.

Peptide-specific cellular and humoral immune activities were measured at 12-week intervals for as long as the samples were available. The peptides used for vaccination and the corresponding immune responses are described in Table 2. One patient (#1) was not eligible because of rapid disease progression. Among the nine patients tested, the augmentation of peptide-specific CTL responses in PBMCs taken after the 12th vaccination by IFN-γ production was observed in eight patients (#2, #4–10), and the augmentation of IgG responses in plasma taken after the 12th vaccination was also observed in eight patients (#2–5, #7–10). Both CTL and IgG responses were boosted in seven of nine the patients tested and CTL or IgG responses to more than two peptides were observed in four and six tested patients, respectively.

All clinical responses were confirmed by an independent review, and were as follows: one complete response, one partial response, two stable disease and six progressive disease (Table 2). A response was recorded on

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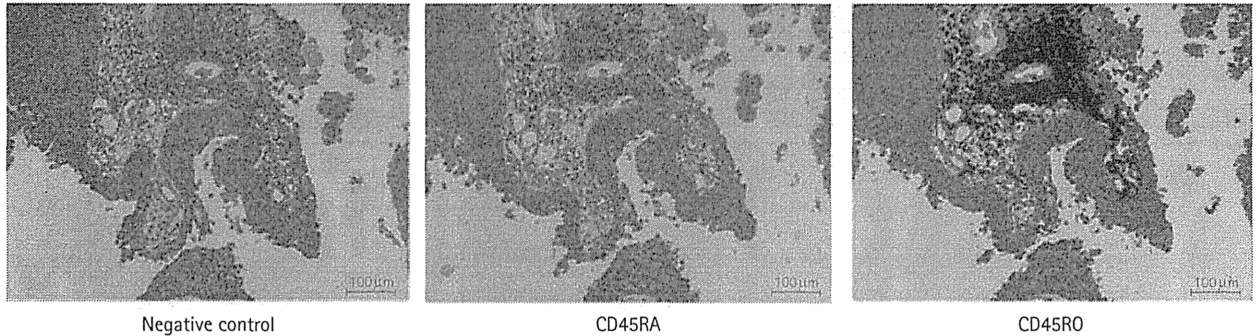
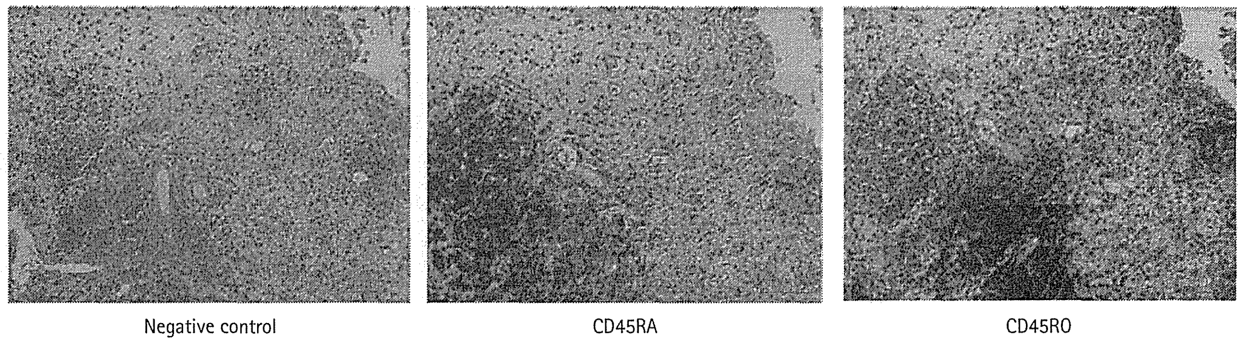
FIG. 2. Representative immunohistochemical stainings of both pre-vaccination tumour regions at the first visit before methotrexate, vinblastine, adriamycin and cisplatin therapy (A) and after the 12th vaccination (B); tumour regions with anti-CD45RO and -CD45RA monoclonal antibodies are shown. The magnification was ×100.

(A) Pre-vaccination



Negative control CD45RA CD45RO

(B) After 12th vaccination



Negative control CD45RA CD45RO

radiological review in four patients. The remaining six patients had disease progressions. None of the six patients who had disease progression had any response to the peptide vaccinations. At the time of analyses, seven patients had died and all patients had progressed except for one patient who had a complete response but died from a cerebral infarction after complete peptide vaccination. The median progression-free survival was 3.0 months (range 0.5–14.1 months). The median overall survival was 8.9 months (range 2.5–29.3 months). Among the four responders, the median progression-free survival and overall survival were 21 (range 2.7–22.4 months) and 24 (range, 9.0–29.3 months), respectively.

It is of note that two patients (#2 and #5) with locally advanced bladder cancer showed obvious clinical responses on kinetic CT images (Fig. 1). To investigate host-tumour interaction, immunohistochemical staining of the biopsied samples taken at the first visit before MVAC therapy and after the 12th vaccination was performed. Immunohistochemical staining at the time of the first visit before MVAC therapy showed that there were a large numbers of tumour cells in the sample, whereas lymphocyte infiltration was limited in stromal lesions. $CD45RA^+$ naive lymphocytes were rare in the stromal lesions, whereas $CD45RO^+$ activated/memory lymphocytes were found around tumour vessels and stromal lesions, but not in tumour sites (Fig. 2A). Immunohistochemical staining after the 12th vaccination showed that there were very few tumour cells in the sample but many lymphoid cells with lymphoid follicles. $CD45RA^+$ naive lymphocytes were massively observed in lymphoid follicles, while $CD45RO^+$ activated/memory lymphocytes were massively observed not only in lymphoid follicles but also in the other lesions (Fig. 2B). These results suggest that PPV induced infiltration of both $CD45RA^+$ and $CD45RO^+$ cells into tumour sites, which in turn resulted in distraction of most of the tumour cells in this patient.

DISCUSSION

No severe adverse events were observed in any of the 10 patients enrolled, although all the patients developed grade 1 or 2 local dermatological reactions at the injection sites. Therefore, in terms of safety, the toxicity of the 12-week regimen of once-weekly PPVs was tolerable and acceptable for patients with MVAC-refractory UC.

With regard to peptide-induced immune reactions, an increase in peptide-specific IFN-γ production in response to at least one of the four vaccinated peptides was observed in most of the post-vaccination PBMCs (eight of nine cases), regardless of the absence ($n = 5$) or reduced levels ($n = 5$) of CTL activity in pre-vaccination PBMCs. Boosted CTL activities in response to all four peptides were seen in the

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post-vaccination samples of the patient with complete remission (#5). Similarly, an increase of peptide-specific IgGs was observed in the post-vaccination plasma of most patients (eight of nine cases). There were more than 10-fold ($n = 7$) and 100-fold ($n = 6$) increases of the IgG levels in the post-vaccination samples, suggesting that clonal expansion of peptide-reactive B cells was induced by this regimen.

These results indicated that both the cellular and humoral responses were well boosted in most patients with UC under this regimen. The profile of positive peptides varied greatly from patient to patient, suggesting that the peptides suitable for use in each patient were different, which is consistent with the previously reported results in other types of cancers [11–15]. This would be because of the heterogeneous nature of the different tumours studied and the immunological diversity of the tumour-reactive CTLs in each patient.

Although cellular immunity is the predominant effector arm of antitumour responses, humoral immunity could also play an important role in host defence against cancer cells [25]. However, the mechanism of antibody production against the small vaccination peptides is unclear. One possible explanation is that pre-existing CD4 T helper type 1 cells specific to the vaccinated peptides recognize peptides loaded on HLA-class IA molecules and so facilitate both CTL induction and IgG production. Alternatively, some peptides may bind both class I and class II HLA and induce activation of CTL and T helper type 1 cells [26]. The biological roles of peptide-reactive IgGs will also need to be clarified in the near future.

This is a phase I trial designed to investigate toxicity and immune responses, but a description of the clinical responses could be important for the next stage of clinical trials. The overall response rate defined by radiological imaging is comparable to those seen in previously reported studies using chemotherapy combinations such as gemcitabine and paclitaxel [27,28]. The median survival time of our 10 patients was somewhat shorter than those reported for patients on chemotherapy regimens [27–29], but the four responders to peptide vaccination showed a median survival time of 24 months, suggesting that PPV has the potential to provide long-term survival in some patients with advanced UC.

In this study, we observed massive infiltration of both $CD45RA^+$ and $CD45RO^+$ cells into tumour sites of a PR patient after PPV, whereas they resided around vessels and connective tissues before the vaccination (at the first visit). We previously reported that PPV induced infiltration of $CD45RO^+$ lymphocytes, but neither $CD8^+$ T cells nor $CD20^+$ B cells, in tumour sites of patients with prostate cancer [24]. In considering CD45RO expression in activated or memory T cells and CD45RA expression in naive T cells [30], PPV induced infiltration of both $CD45RA^+$ and $CD45RO^+$ cells into tumour sites, which in turn resulted in destruction of most tumour cells in this patient. Further studies with other patients' samples will be needed to clarify this issue.

The potential efficacy of 12 consecutive weekly vaccinations with PPV in patients with advanced UC merits further investigation based on the safety and boosted immune responses shown herein.

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CONFLICT OF INTEREST

None declared.

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Abbreviations: **UC**, urothelial carcinoma; **MVAC**, methotrexate, vinblastine, adriamycin and cisplatin; **HLA**, human leucocyte antigen; **CTL**, cytotoxic T lymphocyte; **PPV**, personalized peptide vaccination; **PBMC**, peripheral blood mononuclear cell; **IFN-γ**, interferon-γ.

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Phase I clinical study of a personalized peptide vaccination available for six different human leukocyte antigen (HLA-A2, -A3, -A11, -A24, -A31 and -A33)-positive patients with advanced cancer

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Abstract. The majority of peptide-based cancer vaccines under development are for human leukocyte antigen (HLA)-A2- or -A24-positive patients. To overcome this limitation, we conducted a phase I clinical study of peptide vaccines designed for cancer patients with six different HLA-A types. Eligible patients were required to have failed prior standard cancer therapies and to be positive for the HLA-A2, -A24 or -A3 (A3, A11, A31 and A33) supertype. Three sets of 8 candidate peptides (24 peptides in total) were provided for vaccination to HLA-A2$^+$, HLA-A24$^+$ and HLA-A3$^+$ patients, respectively. Personalization of the vaccination peptides from the candidate pool was made by considering the patients' HLA types and pre-existing levels of IgGs to the candidate peptides. Seventeen patients were enrolled in this study. The peptide vaccinations were well tolerated in all patients with no vaccine-related severe adverse events. Augmentation of cytotoxic T lymphocyte (CTL) or IgG responses specific to the vaccinated peptides was observed in 11 or 10 out of 13 cases tested, respectively. This new type of vaccine is recommended for phase II clinical trial because of its tolerability and the immune responses to the vaccinated peptides.

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Abbreviations: CTL, cytotoxic T lymphocyte; HLA, human leukocyte antigen; PBMCs, peripheral blood mononuclear cells

Key words: personalized, cancer vaccine, peptide, clinical trial, phase I

Introduction

Immunotherapy is one of the most promising modalities for cancer treatment. Identification of T-cell epitopes of cancer antigens enables the development of peptide-based cancer vaccines. More than 200 T-cell epitopes of cancer antigens have been identified, and clinical trials using these peptides are in progress at various institutions with promising results (1,2).

It is well known that recognition of cancer antigens by T-cell receptors is restricted by class I-human leukocyte antigen (HLA) molecules, and epitope peptides recognized by T-cells differ according to the HLA type of the patients (1-5). This fact, together with the presence of many different types of class I-HLAs, hampers the development of peptide-based cancer vaccines at the industrial level (1,2).

Currently, peptide-based cancer vaccines are mainly developed for HLA-A2$^+$ or HLA-A24$^+$ patients and rarely developed for the other types of class I-HLAs (1,2). The approximate frequencies of the HLA-A2 and -A24 populations are as follows: 40 and 60% in Japanese, 50 and 20% in Caucasians, and 30 and 12% in African blacks, respectively (6). Therefore, the currently developed HLA-A2 and -A24 vaccines only cover 76% [40 + 60% x (100 - 40)/100%] of Japanese, 60% of Caucasians and 38% of African blacks, and development of new vaccines for the other types of class I-HLAs is required. After HLA-A2 or -A24, the most frequent class-I HLA types in Japanese are HLA-A26 and -A11 (occurring in approximately 20% of the population), followed by -A31 and -A33 (~15%) (6). HLA-A11, -A31 and -A33, as well as -A3, make up the A3 supertype, and common binding motifs of antigenic peptides for these HLA types have been identified (7).

In the present study, to overcome HLA-based limitations in the development of peptide vaccines, three sets of 8 candidate peptides (24 peptides in total) were provided for vaccination to HLA-A2$^+$, HLA-A24$^+$ and HLA-A3$^+$ patients, respectively. Personalization of the vaccination peptides from the candidate

peptide pool was conducted by considering the HLA types of the patient and the pre-existing levels of IgGs to the candidate peptides (1,2,8-10), and the safety and immunological effects of the vaccination were analyzed.

Patients and methods

Patients. Between April 2007 and September 2008, 17 patients with different types of HLA-A alleles and different types of cancers who visited the outpatient clinic of Kurume University Hospital were enrolled in this study. The study was approved by the Kurume University Ethics Committee and was registered in UMIN-CTR (UMIN000000619). Eligibility criteria of the patients were as follows. i) Patients were pathologically diagnosed with cancer which was refractory to standard cancer therapies; ii) patients possessed target lesions for evaluation of response; iii) patients were positive for HLA-A2, -A3, -A11, -A24, -A31 or -A33; iv) patients had significant levels of plasma IgGs reactive to at least one of the vaccine candidate peptides; and v) any patients who previously received pre-therapies, including chemotherapy, immunotherapy or radiation therapy, started vaccine therapy more than 4 weeks after the last treatment. In the case of 5-FU and 5-FU-related compounds, the vaccine therapy started after a washout period of more than 2 weeks, and patients did not exhibit any remaining anti-tumor effects or adverse effects of pre-therapy at the time of vaccine therapy; vi) patients had an Eastern Cooperative Oncology Group (ECOG) performance status score of 0-2; vii) patients were expected to survive more than 3 months; viii) patients satisfied the following: WBC $\geq$3,000/mm^3, lymphocytes $\geq$1,000/mm^3, Hb $\geq$9.0 g/dl, platelets $\geq$100,000/mm^3, serum creatinine $\leq$1.4 mg/dl and total bilirubin $\leq$1.5 mg/dl; ix) patients were negative for hepatitis virus B/C; x) patients were more than 20 years and less than 80 years of age; and xi) all of the patients provided written informed consent prior to study entry.

The following patients were excluded from the study: i) patients with severe symptoms (active or severe infectious, circulatory, respiratory or kidney disease, immunodeficiency and disturbance of coagulation); ii) patients with a past history of severe allergic reactions; iii) patients who were pregnant or nursing, or who were currently attempting to become pregnant. (This included patients who had failed to use effective contraception during or for at least 70 days after study participation.) and iv) patients who were judged inappropriate for the clinical trial by doctors.

Clinical protocol. This was an open-label phase I study. The primary endpoint of this study was adverse events (evaluation of safety) and the secondary endpoint was immunological effects of group 3 peptides (see Peptides and vaccine preparation). Plasma levels of IgGs specific to the 24 different vaccine candidate peptides were measured for screening. Based on the results of the HLA typing and the IgG screening, peptides showing higher immune responses (a maximum of 4 peptides) were selected from an appropriate group(s) of candidate peptides for injection as reported previously (8-10). If two groups of candidate peptides were appropriate for a patient, a maximum of 2 peptides were selected from each group. The peptides (3 mg/peptide) were subcutaneously injected with incomplete Freund's adjuvant (IFA) once a week for 6 weeks as reported previously (8-10). We investigated immunological responses to the vaccinated peptides after every six vaccinations. After the 6th vaccination, the vaccinated peptides were re-selected according to the results of immunological tests. Physical examination was performed weekly throughout the entire treatment period. Complete blood counts and serum chemistry tests were performed once every 2 weeks. All the vaccinated patients (n=17) were assessed for toxicity. Thirteen patients whose samples were available both pre- and post- (after the 6th) vaccination were assessed for immunological responses. Toxicity was assessed according to the common toxicity criteria for adverse events (CTCAE) version 3.0.

Peptides and vaccine preparation. Three sets of 8 candidate peptides (24 peptides in total) were provided for vaccination to HLA-A2$^+$, HLA-A24$^+$ and HLA-A3$^+$ supertype patients, respectively. The peptide name, 'mother' protein name, amino acid (a.a.) positions in the protein and a.a. sequences are provided in Table I.

The peptides were prepared under the conditions of Good Manufacturing Practice by the PolyPeptide Laboratories (San Diego, CA, USA) and the American Peptide Company (Vista, CA, USA). The safety and immunological effects of the HLA-A2 and -A24 peptides were confirmed in previously conducted clinical studies (8-10). By contrast, the 8 peptides included in the pool for the HLA-A3 supertype were not previously used in clinical trials, although these peptides were previously shown to induce cytotoxic T lymphocyte (CTL) activity *in vitro* in HLA-A3$^+$ supertype cancer patients (11-13).

The peptides were dissolved in the appropriate solvents and stored at -80°C. The stock solutions were diluted with saline, sterilized by filtration and mixed with an equal volume of IFA to make the emulsion preparation. The IFA formulation designated as 'NH2' consisted of sorbitan monooleate (NOFABLE SO-991; NOF Corporation, Tokyo, Japan) 11.4 w/w% and mineral oil (Hicall M-72; Kaneda, Tokyo, Japan) 88.6 w/w%. NOFABLE SO-991 and Hicall M-72 are pharmaceutical-grade products, and their regulatory status meets the requirements of the Japanese Pharmaceutical Excipients and the Japanese Pharmacopeia, respectively.

Peptide-specific CTL response. Screening of peptide-specific CTL precursors was conducted using 30 ml of peripheral blood obtained from each patient. Peripheral blood mononuclear cells (PBMCs) were separated by means of Ficoll-Conray density gradient centrifugation. Peptide-specific CTL responses in PBMCs were detected using a previously reported culture method (14). Briefly, PBMCs (1x10^5 cells/well) were incubated with 10 μM of a peptide in 200 μl of culture medium in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark). The culture medium consisted of 45% RPMI-1640 medium, 45% AIM-V medium (Gibco BRL, Walkersville, MA, USA), 10% fetal calf serum, 100 IU/ml of interleukin (IL)-2 and 0.1 μM MEM non-essential amino acid solution (Gibco BRL). Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) every 3 days.

Table I. Vaccine candidate peptides.

HLA	Peptide name	Mother protein	a.a. Position	a.a. Sequence
HLA-A2				
	SART3-302	SART3	302-310	LLQAEAPRL
	CypB-129	Cyclophilin B	129-138	KLKHYGPGWV
	Lck-246	p56 lck	246-254	KLVERLGAA
	Lck-422	p56 lck	422-430	DVWSFGILL
	ppMAPkkk-432	ppMAPkkk	432-440	DLLSHAFFA
	WHSC2-103	WHSC2	103-111	ASLDSDPWV
	UBE2V-43	UBE2V	43-51	RLQEWCSVI
	HNRPL-501	HNRPL	501-510	NVLHFFNAPL
HLA-A24				
	SART2-93	SART2	93-101	DYSARWNEI
	SART3-109	SART3	109-118	VYDYNCHVDL
	Lck-208	p56 lck	208-216	HYTNASDGL
	Lck-488	p56 lck	488-497	DYLRSVLEDF
	MRP3-1293	MRP3	1293-1302	RYLTQETNKV
	PAP-213	PAP	213-221	LYCESVHNF
	PSA-248	PSA	248-257	HYRKWIKDTI
	EGF-R-800	EGF-R	800-809	DYVREHKDNI
HLA-A3 supertype				
	SART3-511	SART3	511-519	WLEYYNLER
	SART3-734	SART3	734-742	QIRPIFSNR
	Lck-90	p56 lck	90-99	ILEQSGEWWK
	Lck-449	p56 lck	449-458	VIQNLERGYR
	PAP-248	PAP	248-257	GIHKQKEKSR
	PSA-16	PSA	16-24	GAAPLILSR
	IEX1-47	IEX-1	47-56	APAGRPSASR
	β-tubulin5-154	β-tubulin5	154-162	KIREEYPDR

After incubation for 14 days, these cells were harvested and tested for their ability to produce interferon (IFN)-γ in response to T2, CIR-A11, CIR-A24, CIR-A31 or CIR-A33 cells (stable transformants of CIR cells with HLA-A1101, -A2402, -A31012 and -A3303, respectively) that were pre-loaded with either a corresponding peptide or a negative control peptide from human immunodeficiency virus (HIV) as reported previously (8-13). The level of IFN-γ was determined by enzyme-linked immunosorbent assay (limit of sensitivity, 10 pg/ml). All assays were carried out in quadruplicate and were analyzed by the Student's t-test. The peptide-specific IFN-γ production (net value) was estimated as the difference between the IFN-γ production in response to target cells with a corresponding peptide and the IFN-γ production in response to target cells with an HIV peptide; differences of $P \le 0.05$ were considered statistically significant. The IFN-γ production of individual wells in the quadruplicate cultures was considered positive when the net value was >50 pg/ml.

Measurement of peptide-specific IgGs. The peptide-specific IgG levels were measured using a Luminex system (Luminex, Austin, TX, USA) as reported previously (8,10). In brief, plasma was incubated with 25 μl of peptide-coupled color-coded beads for 2 h at room temperature on a plate shaker. After incubation, the mixture was washed with a vacuum manifold apparatus and incubated with 100 μl of biotinylated goat anti-human IgG (γ-chain specific) for 1 h at room temperature. The plate was then washed, followed by the addition of 100 μl of streptavidin-phycoerythrin per well and incubation for an additional 30 min at room temperature on a plate shaker. The bound beads were washed three times, followed by the addition of 100 μl of Tween-20 phosphate-buffered saline into each well. Each sample (50 μl) was then analyzed using the Luminex system.

Results

Patient characteristics. Between April 2007 and September 2008, 17 patients with different types of advanced cancer were enrolled in this study (Table II). There were 15 male and 2 female subjects, with a median age of 70 years (range 53-76). All patients had advanced-stage cancer and were previously treated with and failed to respond to the standard therapy for the particular cancer type, including surgery, chemotherapy, hormone therapy, radiation therapy or a combination of two or more of the above. There were 2 HLA-A2, 8 HLA-A24 and 12 HLA-A3 supertype-positive patients.

Table II. Patient characteristics.

Patient ID	HLA type	Disease	Disease stage	Age/Gender[a]	Previous treatment
D1	A2/A24	Vulvar carcinoma	IVb	76/F	Surgery, chemo
001	A24	Prostate cancer	D2	68/M	Hormone
002	A24/A11	Bladder cancer	IV	63/M	Surgery, chemo
003	A24	Prostate cancer	D1	58/M	Hormone, chemo
004	A24/A33	Penile SCC	III	74/M	Surgery, chemo-radiation
005	A26/A31	Prostate cancer	D1	71/M	Hormone
008	A24	Prostate cancer	D2	75/M	Hormone, chemo
009	A24/A31	Prostate cancer	D2	69/M	Hormone
010	A24/A26	Prostate cancer	C	72/M	Hormone
011	A26/A31	Gallbladder cancer	IV	68/M	Surgery, chemo, tumor-cell vaccine
101	A2/A33	Renal cell carcinoma	IV	54/M	Surgery
301	A11/A33	Prostate cancer	D1	56/M	Surgery, hormone, chemo, radiation
302	A11/A33	Prostate cancer	C	70/M	Hormone, chemo, radiation
303	A11	Prostate cancer	D2	70/M	Hormone
304	A11/A31	Skin SCC	III	74/M	Radiation, chemo
305	A11/A26	Prostate cancer	D2	65/M	Surgery, hormone, chemo, radiation
306	A11/A33	Adorenocortical cancer	IV	53/F	Surgery, chemo

[a]Median age of the patients was 70 years (range 53-76). SCC, squamous cell carcinoma.

Toxicities. The overall toxicities are shown in Table III. The most frequent adverse events were injection site reactions (n=9), tumor site pain (n=7) and limb edema (n=4). All of the adverse events were grade 1 or 2, with the exception of one case of tumor site pain (grade 3). Severe adverse events (grade ≥3) were as follows: increase in serum ALT (n=1, grade 3), increase in serum creatinine (n=2, grade 3), rectal fistula (n=1, grade 3), decrease of hemoglobin (n=2, grade 4; n=2, grade 3), duodenal hemorrhage (n=1, grade 3), hypotension (n=1, grade 4), penile infection (n=1, grade 4), lymphopenia (n=1, grade 3), stricture of the ureter (n=1, grade 4, n=1, grade 3), urticaria (n=1, grade 3) and death (n=1, grade 5). All of the severe adverse events (grade ≥3) observed in this study were evaluated by an independent safety evaluation committee, who concluded that the events were associated with cancer progression.

Peptides and immunological responses. The HLA-A types of the patients (n=17) were HLA-A2 (n=2), -A11 (n=4), -A24 (n=8), -A26 (n=3), -A31 (n=5) and -A33 (n=2). The numbers of peptides vaccinated to the patients were 4 peptides to 8 patients, 3 to 7 patients, and 2 to 2 patients. Post 6th vaccination, blood samples were obtained from 13 of the patients. Since 14 patients received at least six vaccinations, 13 patients were eligible for immunological analysis (Table IV). The median number of vaccinations was 12, with a range of 6-37. Both CTL and IgG responses to the vaccinated peptides were analyzed in the pre- and post- (6th and 12th) vaccination samples (Table IV). The remaining 3 patients withdrew from the trial earlier due to disease progression, terminating treatment after only one, one and four vaccinations, respectively.

When the IgG level of the post-vaccination plasma was more than 1.5-fold higher than that of the pre-vaccination plasma, the increment was considered to be significant. When a significant increment was observed for at least one vaccinated peptide, the specific IgG response was considered to be augmented. Under these criteria, the peptide-specific IgG response was augmented in 8 out of 13 patients after the 6th vaccination and in all 6 patients after the 12th vaccination.

We considered the IFN-γ production of individual wells of the quadruplicate culture to be positive when the net value was >50 pg/ml. If the number of positive wells of the post-vaccination samples increased – or in cases in which the number of positive wells was unchanged between the pre- (before the 1st vaccination) and post-vaccination samples, if the net values of positive wells increased more than 2-fold – then the IFN-γ response was considered to be augmented. Under these criteria, augmentation of CTL activity in response to at least one of the vaccinated peptides after the 6th and 12th vaccination was observed in 11 out of 12 and 2 out of 3 patients tested, respectively. Patient 301 had two different types of HLA-A3 supertypes (HLA-A11 and -A31), and each type-restricted CTL response to the vaccinated peptides was investigated. An augmented CTL response to the Lck-449 peptide was observed in both HLA types, whereas augmentation to the IEX-47 peptide was observed only in an HLA-A33-restricted manner (Table IV). Similarly, patient 306 had two different types of HLA-A3 supertypes (HLA-A11 and -A33), and an augmented CTL response to the SART3-511 and Lck-449 peptides was observed only in an HLA-A33-restricted manner (Table IV). The SART3-109 peptide, which has the ability to induce CTL activity in PBMCs of both HLA-A24 and -A3 supertypes, but not in HLA-A2$^+$

Table III. Adverse events.

Adverse events	No. of patients presenting with each event						
	G1	G2	G3	G4	G5	Total	Frequency (%)
						0	0
Injection site reaction	3	6				9	52.9
ALT, SGPT	1		1			2	11.8
AST, SGOT	2					2	11.8
Creatinine			2			2	11.8
Death not associated with CTCAE – disease progression NOS					1	1	5.9
Dermatology/skin-other, herpes zoster		1				1	5.9
Diarrhea	1					1	5.9
Edema: limb		4				4	23.5
Fatigue	2	1				3	17.6
Fever	1	2				3	17.6
Fistula, GI-rectum			1			1	5.9
Hemoglobin			2	2		4	23.5
Hemorrhage, GI-duodenum, stomach			1			1	5.9
Hypotension				1		1	5.9
Infection-other, penis				1		1	5.9
Lymphopenia			1			1	5.9
Pain-tumor pain	1	5	1			7	41.2
Potassium, serum-high		1				1	5.9
Somnolence		1				1	5.9
Stricture/stenosis, GU-ureter			1	1		2	11.8
Urticaria			1			1	5.9
Vomiting	2	2				4	23.5

patients, was vaccinated to patient 009 (HLA-A24 and -A31). Therefore, CTL activity to the SART3-109 peptide in both an HLA-A24- and -A31-restricted manner was investigated. As a result, an augmented CTL response was observed in both the HLA types, with the HLA-A24-restricted induction being much stronger (Table IV).

Discussion

HLA restriction is the main feature of T-cell-mediated recognition of antigenic peptides on the binding groove of HLA molecules (15). Antigen epitope peptides recognized by T-cells are different in each HLA type (4,5). Therefore, it is difficult to develop peptide-based cancer vaccines applicable for patients with rare types of HLAs at the industrial level.

To overcome the hurdle of HLA restriction, protein vaccines are sometime developed. Protein vaccines usually contain multi-epitope peptides, recognized by both CTLs and helper T-cells, which may cover several different HLA types. However, whole protein vaccines may also contain allergic epitopes, since some of the target molecules for cancer vaccines have been identified as allergens for atopic dermatitis (16). In addition, the protein vaccines have the following disadvantages when compared to peptide vaccines. i) Protein vaccines are biologics rather than chemicals and therefore the industrial cost of chemistry, manufacturing and control is much higher for protein vaccines than for peptide vaccines, and it is difficult to prepare many different types of proteins as candidates for personalized vaccines. ii) The relative molar contents of the epitope peptides in the protein vaccine preparations are generally lower by a factor of several tens compared to that of peptide vaccines. For these reasons, we sought to develop a peptide-based personalized vaccine applicable for virtually all patients with different types of HLAs. Our recent study demonstrated that several vaccine candidate peptides which were originally identified in HLA-A24$^+$ patients, were recognized by CTLs of different types in an HLA-restricted manner (18). Furthermore, HLA-A11, -A31 and -A33, as well as -A3, make up the A3 supertype, and common binding motifs of antigenic peptides for these HLA types have been identified (7). Approximately 95% of Japanese possess at least one of the HLA-A2, -A24 and -A3 supertypes. Our cancer vaccine candidate peptides were derived from commonly expressed antigens in different tissue types and origins of cancer cells (1,2). Therefore, the sets of 8 peptides used for each of the HLA-A2, -A24 and -A3 supertypes in this study could be applicable for the majority of Japanese cancer patients irrespective of their HLA type. These vaccine candidate

Table IV. Peptide-specific IgG responses induced by the peptide vaccination.

Patient ID (HLA type)	Total no. of vaccinations	Vaccinated peptides	Peptide-specific IgG (FIU)		
			Before 1st	Post 6th	Post 12th
001 (A24/A24)	28	SART3-109	5,043	**19,505**	**19,164**
		MRP3-1293	116	**193**	**2,822**
		PAP-213	46	**5,154**	**10,168**
		PSA-248	8,288	**21,420**	**24,132**
002 (A11/A24)	16	SART3-511	71	**333**	**417**
		SART3-734	11	<10	<10
		SART2-93	10	**20**	**154**
		SART3-109	531	450	359
003 (A24/A24)	9	SART2-93	22	22	ND
		SART3-109	945	**7,675**	ND
		lck-488	18	17	ND
		MRP3-1293	16	16	ND
008 (A24/A24)	29	SART3-109	79	**2,826**	**38,887**
		PAP-213	179	**428**	**2,322**
009 (A24/A31)	25	SART3-109	544	527	**18,131**
		SART3-734	242	241	**1,636**
		PAP-248	34	34	38
010 (A24/A26)	8	SART3-109	44	45	ND
		lck-488	98	96	ND
		MRP3-1293	82	84	ND
		PAP-213	69	68	ND
011 (A26/A31)	11	SART3-109	262	242	ND
		SART3-734	373	**627**	ND
		lck-449	204	194	ND
101 (A2/A33)	18	CypB-129	220	105	**22,217**
		SART3-511	802	**1,244**	**2,467**
		SART3-734	54	25	30
301 (A11/A31)	6	SART3-511	260	319	ND
		lck-449	185	239	ND
		IEX1-47	21	<10	ND
302 (A11/A31)	6	SART3-511	10	ND	ND
		Lck-449	290	ND	ND
		IEX1-47	15	ND	ND
303 (A11/A11)	6	SART3-511	347	**1,219**	ND
		lck-449	201	**587**	ND
		β-tubulin5-154	93	86	ND
304 (A11/A31)	6	SART3-734	346	267	ND
		lck-449	312	251	ND
		PAP-248	27	25	ND
305 (A11/A26)	37	SART3-511	231	186	**744**
		SART3-734	68	58	**243**
		lck-90	73	53	**21,307**
		lck-449	143	124	**32,987**
306 (A11/A33)	12	SART3-511	107	52	ND
		SART3-734	38	<10	ND
		lck-90	48	40	ND
		lck-449	85	**136**	ND

Values in bold print indicate significant augmentation (>1.5-fold higher than that of the pre-vaccination plasma) of IgG response. FIU, fluoresence intensity unit; ND, not determined.

Table V. Peptide-specific CTL responses induced by the peptide vaccination.

Patient ID (HLA)	Vaccinated peptides (for HLA-A type)	HLA-restriction	Peptide-specific IFNγ production (ng/ml)[a]		
			Before 1st	Post 6th	Post 12th
001	SART3-109 (A24)	A24	- (0)	182, 273 (2)	ND
(A24/A24)	MRP3-1293 (A24)	A24	- (0)	96,267,643,60 (4)	ND
	PAP-213 (A24)	A24	- (0)	- (0)	ND
	PSA-248 (A24)	A24	- (0)	1401 (1)	ND
002	SART3-511 (A11)	A11	- (0)	- (0)	ND
(A11/A24)	SART3-734 (A11)	A11	- (0)	- (0)	ND
	SART2-93 (A11)	A11	- (0)	365 (1)	ND
	SART3-109 (A11/A24)	A11	- (0)	- (0)	ND
	SART3-109 (A11/A24)	A24	ND	ND	ND
003	SART2-93 (A24)	A24	- (0)	75, 58 (2)	ND
(A24/A24)	SART3-109 (A24)	A24	- (0)	- (0)	ND
	lck-488 (A24)	A24	- (0)	- (0)	ND
	MRP3-1293 (A24)	A24	- (0)	- (0)	ND
008	SART3-109 (A24)	A24	- (0)	281,62 (2)	ND
(A24/A24)	PAP-213 (A24)	A24	- (0)	- (0)	ND
009	SART3-109 (A24/A31)	A24	- (0)	1564, 889, 171 (3)	1261, 1511, 141 (3)
(A24/A31)	SART3-109 (A24/A31)	A31	- (0)	84 (1)	46 (1)
	SART3-734 (A31)	A31	- (0)	- (0)	75 (1)
	PAP-248 (A31)	A31	- (0)	52 (1)	108 (1)
010	SART3-109 (A24)	A24	- (0)	- (0)	ND
(A24/A26)	lck-488 (A24)	A24	- (0)	- (0)	ND
	MRP3-1293 (A24)	A24	- (0)	- (0)	ND
	PAP-213 (A24)	A24	- (0)	1346 (1)	ND
011	SART3-109 (A31)	A31	- (0)	- (0)	ND
(A26/A31)	SART3-734 (A31)	A31	- (0)	177 (1)	ND
	lck-449 (A31)	A31	- (0)	- (0)	ND
101	CypB-129 (A2)	A2	- (0)	- (0)	- (0)
(A2/A33)	SART3-511 (A33)	A33	- (0)	101, 41 (2)	107, 99 (2)
	SART3-734 (A33)	A33	- (0)	- (0)	- (0)
301	SART3-511 (A11/A31)	A11	- (0)	- (0)	ND
(A11/A31)	SART3-511 (A11/A31)	A31	- (0)	- (0)	ND
	lck-449 (A1/A31)	A11	- (0)	136, 157 (2)	ND
	lck-449 (A1/A31)	A31	- (0)	213 (1)	ND
	IEX1-47 (A11/A31)	A11	- (0)	- (0)	ND
	IEX1-47 (A11/A31)	A31	- (0)	60 (1)	ND
302	SART3-511 (A11/A31)	A11	ND	ND	ND
(A11/A31)	SART3-511 (A11/A31)	A31	ND	ND	ND
	Lck-449 (A11/A31)	A11	ND	ND	ND
	Lck-449 (A11/A31)	A31	ND	ND	ND
	IEX1-47 (A11/A31)	A11	ND	ND	ND
	IEX1-47 (A11/A31)	A31	ND	ND	ND
303	SART3-511 (A11)	A11	- (0)	- (0)	ND
(A11/A11)	lck-449 (A11)	A11	- (0)	1165, 1557, 719 (3)	ND
	β-tubulin5-154 (A11)	A11	- (0)	- (0)	ND
304	SART3-734 (A11/A31)	A11	- (0)	- (0)	ND
(A11/A31)	SART3-734 (A11/A31)	A31	ND	ND	ND
	lck-449 (A11/A31)	A11	- (0)	- (0)	ND
	lck-449 (A11/A31)	A31	ND	ND	ND
	PAP-248 (A11/A31)	A11	- (0)	- (0)	ND
	PAP-248 (A11/A31)	A31	ND	ND	ND

Table V. Continued.

Patient ID (HLA)	Vaccinated peptides (for HLA-A type)	HLA restriction	Peptide-specific IFNγ production (ng/ml)[a]		
			Before 1st	Post 6th	Post 12th
305	SART3-511 (A11)	A11	- (0)	ND	- (0)
(A11/A26)	SART3-734 (A11)	A11	- (0)	ND	- (0)
	lck-90 (A11)	A11	- (0)	ND	- (0)
	lck-449 (A11)	A11	- (0)	ND	- (0)
306	SART3-511 (A11/A33)	A11	- (0)	- (0)	ND
(A11/A33)	SART3-511 (A11/A33)	A31	- (0)	- (0)	ND
	SART3-734 (A11/A33)	A11	- (0)	- (0)	ND
	SART3-734 (A11/A33)	A31	- (0)	- (0)	ND
	lck-90 (A11/A33)	A11	- (0)	- (0)	ND
	lck-90 (A11/A33)	A31	- (0)	- (0)	ND
	lck-449 (A11/A33)	A11	- (0)	- (0)	ND
	lck-449 (A11/A33)	A31	- (0)	517 (1)	ND

[a]Values of IFNγ production (pg/ml) in the positive wells are indicated. The number of positive wells in the quadruplicate cultures is also shown in parenthesis. ND, not determined.

peptides could be applicable for 95-99% of Asians, 80-83% of Caucasians, 80-84% of Spanish, 81-88% of Indians and 52-66% of Blacks. Therefore, these sets of peptide vaccines may be applicable for large numbers of cancer patients with different HLA-A types worldwide.

Augmentation of CTL activity to at least one of the vaccinated peptides was observed in 11 out of 12 patients after the 6th vaccination and in 2 out of 3 cases after the 12th vaccination. The frequency of CTL augmentation was higher than in any of our previously conducted clinical trials of personalized peptide vaccines for advanced cancer patients (8-10). This may have been at least partly due to the IFA used in the trials. In this trial we used a new formulation of IFA which we designated 'NH2', whereas previously conducted trials used the commercially available ISA51 made by Seppic (Paris, France). Indeed, we found that 'NH2' was more effective than ISA51 with regard to CTL induction specific to peptides in murine models (Iseki *et al*, unpublished data).

We previously reported that the SART3-109 peptide induces *in vitro* CTL activity restricted, not only to HLA-A24, but also to the HLA-A3 supertype, but not CTL activity restricted to HLA-A2 molecules (18). We also reported that all 8 peptides used for the HLA-A3 supertype induce *in vitro* CTL activity restricted to HLA-A11, -A31 and -A33 molecules (11-13). Subsequently, we investigated whether these results could be replicated *in vivo* in 3 patients (patients 009, 301 and 306) whose PBMCs were available for study. Indeed, the results showed that the SART3-109 peptide induced CTL activity restricted to both HLA-A24 and -A31 molecules, while the Lck-449 peptide induced CTL activity restricted to both HLA-A11 and -A31 molecules, in agreement with the results from the *in vitro* assays. To our knowledge, this is the first study to show CTL induction restricted to two different HLA molecules in clinical trials. Similarly, this could be the first report of peptide vaccination to cancer patients with the HLA-A3 supertype.

All of the subjects enrolled in this study had failed to respond to various types of standard therapies, and the majority of the patients could not continue the 2nd cycle of vaccination after completion of the 1st cycle. In addition, this was a phase I study designed to investigate the safety of and immune responses to a new type of personalized peptide vaccination applicable for six different HLA-A types. Therefore, the clinical effects of the vaccine were not evaluated in this study.

In conclusion, this phase I clinical study of a personalized peptide vaccine for HLA-A2, -A24 and -A3 supertype (A3, A11, A31 and A33)-positive cancer patients confirmed the safety and immunological effects of the vaccination. These results suggest that this new type of vaccine is applicable for the majority of cancer patients in Japan and other countries.

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Phase II Study of Personalized Peptide Vaccination for Castration-Resistant Prostate Cancer Patients Who Failed in Docetaxel-Based Chemotherapy

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BACKGROUND. Docetaxel-based chemotherapy (DBC) showed limited clinical efficacy for castration-resistant prostate cancer (CRPC) patients. To explore cancer vaccine as a new treatment modality, we conducted a phase II study of personalized peptide vaccine (PPV) for DBC-resistant CRPC patients.
METHODS. Twenty DBC-resistant CRPC patients and 22 patients with no prior DBC, as a control, were treated with PPV using peptides chosen from 31 peptides in patients, respectively. Cytokines, inflammatory markers, and immune responses were measured as candidate biomarkers. DBC-resistant CRPC patients without PPV was set as a historical control for evaluation of clinical benefit of PPV.
RESULTS. Median overall survival (OS) time from the first vaccination was 14.8 months or not reached in DBC-resistant CRPC patients and patients with no prior DBC (log-rank; $P = 0.07$), respectively. Median OS time from the first day of progression disease was 17.8 and 10.5 months in DBC-resistant CRPC patients receiving PPV and those with no PPV ($P = 0.1656$), respectively. Elevated IL-6 levels before vaccination was an unfavorable factor for OS of DBC-resistant CRPC patients ($P = 0.0161$, hazard ratio (HR): 0.024, 95% CI:0.001–0.499) as well as all 42 patients with PPV($P = 0.0011$, HR: 0.212, 95% CI:0.068–0.661) by multivariable analysis.
CONCLUSIONS. Further clinical study of PPV is recommended for DBC-resistant CRPC patients, because of the safety and possible prolongation of MST. Control of elevated IL-6 by combined therapy may provide much better clinical outcome. *Prostate 72:834–845, 2012.* © 2011 Wiley Periodicals, Inc.

KEY WORDS: **personalized peptide vaccine; prostate cancer; docetaxel; overall survival**

INTRODUCTION

Castration-resistant prostate cancer (CRPC) is the second-most common cause of cancer-related death in men in the developed world [1,2]. For patients with metastatic prostate cancer, androgen deprivation therapy improves symptoms, but patients invariably develop progressive disease (PD). In the 1990s, the US

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Food and Drug Administration (FDA) approved mitoxantrone and corticosteroids for use in prostate cancer after a demonstrated improvement in palliative benefit over steroids alone [3,4]. In 2004, two large, randomized, phase III trials with docetaxel-based chemotherapy (DBC) showed an improvement in overall survival (OS) as well as patients' reported outcomes [5,6]. These studies changed the goal of treatment in CRPC patients from pure palliation to a survival benefit and represent a milestone in the treatment of the disease. Although DBC represents the most active chemotherapy for first-line treatment of metastatic CRPC, all patients experience disease progression and the median survival benefit with DBC is only 2–3 months. Currently, there is no standard treatment and median OS of second-line approaches after a therapy with DBC are in the range of 12 months [7]. Clearly, the prognosis is very poor, and new treatments that might favorably affect survival for CRPC patients with progression after DBC are obviously needed.

Prostate cancer arises in a relatively unique organ and may express a number of antigens against which an immune response can be generated. Several of these agents have now demonstrated a significant survival benefit in randomized controlled clinical trials for CRPC patients, and Sipuleucel-T (Provenge, Dendreon Corporation, Seattle, WA) which is a fusion protein between the target antigen [prostatic acid phosphatase (PAP)] and granulocyte monocyte colony stimulating factor (GM-CSF), was approved for CRPC patients by the FDA in 2010. However, the survival benefit of this immunotherapy for CRPC patients with progression after DBC has been under investigation.

Personalized peptide vaccine (PPV) is a multiple peptide vaccine regimen planned according to the pre-existing immunity that could prolong OS of patients with advanced cancer. Under PPV treatment, each patient was tested for their immunological reactivity to many different peptides capable of inducing cytotoxic-T-lymphocyte (CTL) responses. The peptides were derived from a number of targets, including prostate-specific antigen (PSA), PAP, prostate-specific membrane antigen (PSMA), multidrug resistance protein, and a variety of other epithelial tumor antigens. Each patient was immunized with 2–4 peptides on the basis of the reactivity panel, since immune responses to individual peptides are usually quite heterogeneous. The most unique aspect of PPV is the "personalized" selection of antigen peptides ideal for individual patients in consideration of the pre-existing host immunity before vaccination. In view of the heterogeneity and complexity of host immune responses and/or tumors, this approach seems to be more rational, rather than vaccination with non-personalized "universal" tumor antigens. Based on the current paradigm that the adaptive immune system composes of limited size and composition, in which individual cells constantly compete with each other, "inconvenient" immune responses induced by non-personalized antigens that are either non-specific to tumor cells or ineffective for tumor cell killing may cause suppression of pre-existing beneficial immunity, which may lead to poor prognosis in vaccinated patients. Indeed, in our previous clinical trials with non-personalized vaccine regimens, some advanced cancer patients showed a shorter survival than expected, possibly because of the inhibition of pre-existing host immunity [8,9]. In contrast, our recent randomized trials of PPV in consideration of the pre-existing host immunity in individual patients have clearly demonstrated clinical benefit to the CRPC patients [10]. To preliminarily investigate the efficacy and safety of the PPV in CRPC patients while evaluating progression status with or without prior DBC, we prospectively undertook a non-randomized, open-label phase II trial.

PATIENTS AND METHODS

Eligibility

Patients were eligible for inclusion in the study, if they had a histological diagnosis of prostate adenocarcinoma and PD by clinical, radiological, or PSA-based criteria, despite adequate medical or surgical castration therapy with or without prior DBC, and showed positive humoral responses to at least two of the 31 different, candidate peptides, determined by both human leukocyte antigen (HLA)-class IA types and the titers of IgG against each peptide. Any number of previous hormonal therapies was allowed. Patients were required to wait at least 4 weeks for entry into the study after the completion of prior chemotherapy, radiation therapy, or a change in hormonal therapy. Anti-androgen therapy was discontinued for at least 4 weeks before enrollment for patients receiving flutamide, and 6 weeks for those receiving bicalutamide. Additional inclusion criteria included age $\geq$20 years; Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1; positive status for HLA-A2, -A24, -A3 super type (-A3, -A11, -A31, and -A33) or -A26; life expectancy of at least 12 weeks; negative status for hepatitis virus B and C; adequate hematologic, hepatic, and renal function. Exclusion criteria included pulmonary, cardiac, or other systemic diseases; an acute infection; a history of severe allergic reactions; other inappropriate conditions for enrollment judged by clinicians.

The protocol was approved by the Kurume University Ethical Committee, and was registered in UMIN-CTR (UMIN000003028). After full explanation of the protocol, written informed consent was obtained from all patients before enrollment.

Study Design and Treatment

This study was a non-randomized, open-label, phase II study and the primary and secondary endpoints were OS, and to evaluate immunological activity and safety in CRPC patients under treatment with PPV, respectively. OS was calculated from date of start of vaccination to any causes of death.

Thirty one peptide candidates were prepared under conditions of Good Manufacturing Practice using a Multiple Peptide System (San Diego, CA) and American Peptide Company (Vista, CA). The candidate peptides consisted of the following 31: SART3 $_{302–310}$, SART3 $_{309–317}$, Lck $_{246–254}$, WHSC2 $_{141–149}$, UBE2V $_{43–51}$, UBE2V $_{85–93}$, and HNRPL $_{140–148}$ for patients with HLA-A2; SART2 $_{93–101}$, SART2 $_{161–169}$, Lck $_{208–216}$, Lck$_{486–494}$, Lck $_{488–497}$, MRP3 $_{503–511}$, MRP3 $_{1293–1302}$, PAP $_{213–221}$, PSA $_{248–257}$, PSMA $_{624–624}$,EZH2 $_{735–743}$, EGF-R $_{800–809}$, and PTH-rP $_{102–111}$ for patients with HLA-A24; SART3 $_{511–519}$, SART3 $_{734–742}$, Lck $_{90–99}$, Lck $_{449–458}$, and PAP $_{248–257}$ for patients with HLA-A3 super type; SART3 $_{109–118}$ for patients with HLA-A24, -A3 super type or -A26; WHSC2 $_{103–111}$ for HLA-A2, -A3 super type or -A26; ppMAPkkk $_{432–440}$ for patients with HLA-A2 or -A26; HNRPL $_{501–510}$ for patients with HLA-A2 or -A26; CypB $_{129–138}$ for patients with HLA-A2 or -A3 super type; Lck $_{422–430}$ for patients with HLA-A2 or -A3 super type. Original proteins of the employed peptides, except for Lck and MRP3, are ubiquitously expressed on various tissues and organs with preferential expression in malignant cells [11,12]. The lck is expressed on metastatic cancer cells [13], although originally identified as a T cell-specific tyrosine–kinase. The MRP3 is an ATP-binding cassette transporter related to multi-drug resistance of cancer cells [14].

The safety and immunological effects of these 31 peptides had been confirmed in conducted clinical trials [10,15–22].

The selection of the right peptides for vaccination to individual patients were based on the results of HLA typing and peptide-specific IgG titers to each of the 31 different vaccine candidates as reported previously [10,15–22]. Selected peptides were mixed with incomplete Freund's adjuvant (Montanide ISA-51VG; Seppic, Paris, France), and a maximum of four peptides of 1.5 ml emulsion each at a dose level of 3 mg/peptide were injected subcutaneously into the thigh or armpit area once a week for six times. The Montanide ISA51VG was used as an adjuvant in the current study, since it is the most popular in clinical use to induce cellular immunity and has been employed in the majority of peptide vaccine trials in the world [23].

After the first cycle of six vaccinations of up to four antigen peptides, the antigen peptides were re-selected according to the titers of peptide-specific IgG at every cycle of six vaccinations and administered at 2, 3, or 4 week intervals until unacceptable toxicity or withdrawal of consent.

Assessment of Clinical Activity

A complete survey of medical history, physical examination, routine laboratory studies, and serum PSA test were performed prior to treatment, and tests were repeated at every six vaccinations. To investigate biomarkers for OS, C-reactive protein (CRP), serum amyroid A (SAA), and interleukin (IL)-6 in plasma at base line were examined by enzyme-linked immunosorbent assay (ELISA) using the kits from R&D systems (Minneapolis, MN), Invitrogen, and eBioscience (San Diego, CA), respectively. Multiplexed bead-based Luminex assays were used to measure IL-6. Frozen plasma samples were thawed, diluted, and assayed in duplicate in accordance with the manufacturer's instructions. All patients underwent relevant radiologic studies and bone scans every 6 months. Outcomes were assessed by post-therapy changes in serum PSA and by computed tomography (CT) or magnetic resonance imaging (MRI) of measurable disease symptoms if present at the baseline. Post-therapy decreases in PSA level of $\geq$50% were defined as partial responses (PR) and confirmed by two separate measurements $\geq$4 weeks apart. Post-therapy decreases of less than 50% or increases of less than 25% from the baseline were interpreted as stable disease (SD) [24]. For measurable disease symptoms, Response Evaluation Criteria in Solid Tumors was used [25]. PD was defined as radiological progression, or if defined using PSA level alone, three consecutive increases in PSA level and 125% of the baseline PSA value. Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0 (NCI-CTC Ver4).

Measurement of Humoral and T-Cell Responses Specific to the Vaccinated Peptides

The humoral responses specific to the vaccinated peptides were determined by peptide-specific IgG levels using a Luminex system (Luminex, Austin, TX), as reported previously [26]. If the titers of peptide-specific IgG in the post-vaccination plasma were

more than twofold higher than those in the pre-vaccination plasma, the changes were considered to be significant. If a significant increase was observed in at least one vaccinated peptide, the specific humoral response was considered to be augmented.

Although T-cell subsets using flowcytometry was not analyzed in this study, T-cell responses specific to the vaccinated peptides were evaluated by INF-γ ELISPOT using peripheral blood mononuclear cells (PBMCs), which were separated from peripheral blood (30 ml) by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare; Uppsala, Sweden) and stored frozen until analysis. After thawing, PBMCs (2.5×10^4 cells/well) were incubated in 384-well microculture plates (IWAKI, Tokyo, Japan) with 25 μl of medium (OpTmizer™ T Cell Expansion SFM; Invitrogen, Carlsbad, CA) containing 10% FBC (MP Biologicals, Solon, OH), IL-2 (20 IU/ml; Serotec, Oxford, UK), and each peptide (10 μM). Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) after culturing for 3 days. After incubating for the following 6 days, the cells were harvested and tested for their ability to produce interferon (IFN)-γ in response to either the corresponding peptides or a negative control peptide human immunodeficiency virus (sequences: SLYNTYATL for HLA-A2; RYLRQQLLGI for HLA-A24; RLRDLLLIVTR for HLA-A3 supertype; EVIPMFSAL for HLA-A26). Antigen-specific IFN-γ secretion after an 18 hr incubation was determined by ELISPOT, according to the manufacturer's instructions (MBL, Nagoya, Japan). All assays were carried out in triplicate and analyzed with an ELISPOT reader (CTL-ImmunoSpot S5 Series; Cellular Technology Ltd, Shaker Heights, OH). Antigen-specific T-cell responses were evaluated by the difference between the spot numbers in response to the corresponding peptide and those to the control peptide; differences of threefold were considered significant. If the spot numbers in response to the corresponding peptide in the post-vaccination PBMCs were more than threefold higher than those in the pre-vaccination PBMCs, the changes were considered to be significant. If a significant increase was observed in at least one vaccinated peptide, the specific T-cell response was considered to be augmented.

Statistical Analysis

Demographics were compared between groups using χ^2 test or Fisher's exact test for categorical variables and the Student's *t*-test for continuous variables. Probabilities of progression free survival (PFS) and OS in patients with PPV were estimated from the first date of peptide vaccination to PD or death using Kaplan–Meier methods. In comparison of OS between the patients with PPV with prior DBC and PD patients as the matched control cohort, OS were estimated from the date of PD after DBC to death in order to assess uniformly. All analyses are by intent to treat. Surviving patients were censored at October 7, 2010, with follow-up of 97% complete. Patients lost to follow-up are censored at the date last known to be alive. Cox proportional hazard regression analysis was used to develop the univariate and multivariate models describing the association of the independent variables with OS. Independent variables analyzed included age, EOCG performance status, lymphocyte counts, PSA, CRP, SAA, IL-6, prior DBC status, IgG responses, and T-cell responses. All baseline parameters in the models were analyzed as dichotomous variables using cut-off values. Data were analyzed using GraphicPad Prism software (GraphPad Software, San Diego, CA) and the StatView® program (SAS Institute Inc., Cary, NC). A two-sided significance level of 5% was considered statistically significant.

RESULTS

Patients' Characteristics

Between November 2008 and April 2010, 42 patients with CRPC (20 with prior DBC and 22 without prior DBC) were enrolled into this study. All patients with DBC received 60–75 mg/m^2 docetaxel and repeated every 3–4 weeks in combination with oral prednisone (10 mg/day) or dexamethasone (2 mg/day). Median cycle of docetaxel was 6.5 and failed in treatment. Baseline patient characteristics were well balanced between two study cohorts. No statistically significant differences existed between the groups excluding prior DBC. There was no correlation between Gleason score and pre-vaccine PSA doubling times. Before the enrollment all patients received androgen deprivation therapy using luteinizing hormone-releasing hormone (LH-RH) analogue (or had previously undergone castration) or anti-androgen as an initial or secondary hormonal therapy. All patients were eligible and assessable. The control group at the Dokkyo Medical University Koshigaya Hospital represented 17 patients initiating DBC between September 2007 and August 2010. Table I shows patients' characteristics of the study and control group at the Dokkyo Medical University Koshigaya Hospital. The study cohort with prior DBC and control group were well matched regarding median age, ECOG performance status, and median cycle of DBC (6.5 cycles vs. 7 cycles). The study cohort had a higher level of median PSA levels and higher metastatic sites than the control group.

TABLE I. Patient Demographics and Clinical Characteristics

	PPV				Matched control PD after DBC (n = 17)	
	Without prior DBC (n = 22)		With prior DBC (n = 20)			
Characteristics	No. of Patients	%	No. of Patients	%	No. of Patients	%
Age, years						
Median	70.5		70		71	
Range	53–87		61–81		54–80	
ECOG performance status						
0	22	100	17	85	15	88
1	0	0	3	15	2	12
HLA typing						
A24	16	73	13	65	—	—
A2	4	18	4	20	—	—
A3 super type	2	9	3	15	—	—
PSA, ng/ml						
Median	23.4		87.8		14.7	
Range	0–1,920		4.2–1,508		0.016–317	
PSA doubling time, months						
Median	2.7		3.4		—	—
Range	0.5–36		1.4–60		—	—
Lymphocyte, 1,400 μl^{-1}						
Low	8	36	10	50	—	—
High	14	64	10	50	—	—
CRP, 3,000 ng/ml						
Low	11	50	8	40	—	—
High	11	50	12	60	—	—
SAA, 20,000 ng/ml						
Low	13	59	3	15	—	—
High	9	41	17	85	—	—
IL6, 2 pg/ml						
Low	19	86	15	75	—	—
High	3	14	5	25	—	—
Gleason score						
6	1	4	2	10	0	0
7	6	28	6	30	4	23
8	3	14	1	5	3	18
9	10	46	8	40	8	47
10	1	4	2	10	2	12
Unknown	1	4	1	5	0	0
Site of metastasis						
No	3	14	0	0	4	23
Bone only	7	32	9	45	7	42
Bone and nodal/organ	10	46	9	45	2	12
Nodal/organ	2	8	2	10	4	23
Cycle of DBC						
Median	—		6.5		7	
Range	—		1–27		2–19	

PPV, personalized peptide vaccination; DBC, docetaxel-based chemotherapy; PD, progression disease; ECOG, Eastern Cooperative Oncology Group; HLA, human leicocyte antigen; PSA, prostate-specific antigen; CRP, C reactive protein; SAA, serum amyroid A; IL6, interleukin 6.