TABLE IV. (*Continued*)

Dose of peptide	Pts No.	Peptide	Anti-peptide IgG response (FIU)[a] Pre	Post (fourth)	Post (after sixth)	Increased response (after sixth)	Anti-peptide cellular response (pg/ml)[b] Pre	Post (fourth)	Post (after sixth)	Increased response (after sixth)
	10	Lck-486	826	1,632	16,376	Positive	127	ND	7,014	Positive
		Lck-488	21	22	48	—	117	227	115	—
		MRP3-1,293	21	22	24	—	ND	109	ND	—
		PAP-213	15	15	60	Positive	189	ND	285	—
	11	Lck-208	19	18	21	—	211	54	ND	—
		Lck-486	434	349	105	—	ND	ND	ND	—
		Lck-488	12	12	12	—	ND	ND	5,258	Positive
		PTHrP-102	102	99	135	—	ND	2,991	2,934	Positive
	12	Lck-486	392	549	348	—	ND	ND	1,136	Positive
		Lck-488	87	96	64	—	ND	ND	ND	—
		PSA-248	157	2,653	18,163	Positive	ND	ND	ND	—
		SART3-109	76	87	58	—	ND	ND	794	Positive
5 mg	13	Lck-486	183	231	861	Positive	184	103	104	—
		PAP-213	39	35	8,490	Positive	232	ND	ND	—
		SART2-93	56	49	51	—	59	215	ND	—
		SART3-109	31	31	38	—	391	ND	165	—
	14	Lck-486	162	120	2,950	Positive	185	348	126	—
		MRP3-1293	29	27	149	Positive	97	104	ND	—
		SART2-161	16	17	27	—	178	200	263	—
		SART3-109	23	20	108	Positive	1,285	117	1,024	—
	15	Lck-486	809	837	916	—	1,339	ND	ND	—
		MRP3-1293	710	543	550	—	251	ND	ND	—
		SART2-161	72	46	57	—	ND	ND	55	—
		SART3-109	311	248	236	—	100	ND	110	—

[a]Values indicate fluorescence intensity unit (FIU) of IgG antibodies reactive to each peptide.
[b]Values indicate the mean of specific interferon-γ production in positive wells reactive to each peptide.

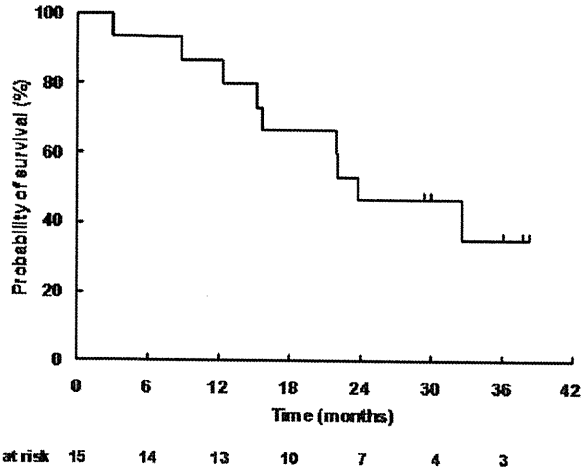

Fig. 1. Kaplan–Meier estimates of overall survival for 15 patients treated by personalized peptide vaccination with low-dose estramustine. Median overall survival is 23.8 months.

because of both widespread grade 2 skin reactions and patients' own requests. Subsequently, we calculated MAD as 8.643 mg/4 peptides in this study. Therefore, considering the adverse events, tolerability, and immune responses, the 3 mg/1.5 mL/peptide dose of PPV will be recommended for further clinical trials.

In the present study, CTL responses measured by IFN-γ release assay and IgG responses were enhanced in 10/15 (66.7%) and 7/15 (46.7%) of the examined patients, respectively, and in the PSA response, CR and PR was one patient each (6.7%) and PD was two patients (13.3%) after the sixth vaccination. In addition, the long-term (23.8 months) median survival time after combination therapy with PPV and low-dose EMP observed in the extension study indicated that this treatment suppresses tumor growth. However, the exact mechanism of this interaction is unclear and further studies are needed.

In conclusion, the results of safety, immune responses, and improved overall survival without MTD, as well as the consistency between these results and the data from our previous trials [4,19,27], could lead to us to the next phase of randomized clinical trial wherein we can confirm the survival benefit of such personalized immunotherapy in HLA-A24 positive patients with CRPC.

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Characteristics of severe adverse events after peptide vaccination for advanced cancer patients: Analysis of 500 cases

KAZUMI YOSHIDA[1], MASANORI NOGUCHI[2,6], TAKASHI MINE[5], NOBUKAZU KOMATSU[1], SHIGERU YUTANI[1], TAKATO UENO[3], HIROAKI YANAGIMOTO[8], KOUICHIROU KAWANO[4], KYOGO ITOH[1,7] and AKIRA YAMADA[7]

Departments of [1]Immunology and Immunotherapy, [2]Urology, [3]Internal Medicine and [4]Obstetrics and Gynecology, Kurume University School of Medicine; [5]Multidisciplinary Cancer Center, Kurume University Hospital; Divisions of [6]Clinical Research and [7]Cancer Vaccine Development, Kurume University Research Center for Innovative Cancer Therapy, Kurume; [8]Department of Surgery, Kansai Medical University, Osaka, Japan

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Abstract. The purpose of this study was to investigate severe adverse events (SAEs) after therapeutic peptide vaccination for advanced cancer patients. We investigated SAEs following personalized peptide vaccinations in 500 advanced cancer patients, including 174 prostate, 74 colon, 51 pancreatic and 43 gastric cancer patients. The number of vaccination cycles varied widely, from 3 to 112. The severity of adverse events was scored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3, and events with a grade of >3 were defined as SAEs and were evaluated by the Institutional Safety Evaluation Committee. A total of 215 SAEs in 102 patients were recorded during the vaccine trials. The main causes for these events were cancer progression (152 SAEs in 78 patients), combined cancer treatments other than vaccination (35 in 21 patients), diseases other than cancer (20 in 19 patients), peptide vaccines (6 in 6 patients) and suicide (1 in 1 patient). The 6 vaccine-related SAEs, all grade 3, consisted of skin reactions at each injection site, cellulitis around the injection site, edemas of the head and neck regions, colitis, rectal bleeding and bladder-vaginal fistulae. Both cellular and humoral responses to the vaccinated peptides were highly boosted in all 6 of these patients, indicating the involvement of augmented immune responses in these SAEs. The clinical responses in these 6 patients consisted of 2 partial responses and 4 stable diseases. The majority of SAEs after peptide vaccination for advanced cancer patients were caused by cancer progression. The appearance of vaccine-related SAEs, except inflammatory injection site reactions, was unexpected, and fortunately the incidence was very low. Our results suggest that physicians should be on guard for these rare SAEs associated with augmented immune responses.

Correspondence to: Professor Akira Yamada, Cancer Vaccine Development Division, Kurume University Research Center for Innovative Cancer Therapy, 67 Asahi-machi, Kurume 830-0011, Japan
E-mail: akiymd@med.kurume-u.ac.jp

Key words: severe adverse events, peptide vaccine, advanced cancer

Introduction

The field of therapeutic cancer vaccines for advanced cancer patients is currently in an active state of clinical investigations. Many clinical trials of therapeutic cancer vaccines have demonstrated their tolerability, based on the absence or rarity of severe adverse events (SAEs) caused by the vaccination (1-10). To our knowledge, however, there has been no detailed study of SAEs after therapeutic peptide vaccines. Indeed, certain randomized trials of tumor cell-based or idiotype vaccines have shown a detrimental effect on the vaccine arm, suggesting that cancer vaccines are not always safe (11-13).

In order to better understand the safety of cancer vaccines, we analyzed the records of a total of 500 advanced cancer patients who received personalized peptide vaccinations between October 2000 and October 2009. SAEs other than injection site reactions were rare, but were also documented.

Materials and methods

Patients. Between October 2000 and October 2008, 500 patients positive for HLA-A24, -A2, or -A3 supertypes with various types of advanced cancer took part in phase I, I/II and II studies for personalized peptide vaccinations after providing their written informed consent. The advanced cancers originated from the prostate (n=174 patients), colon and rectum (n=74), pancreas (n=51), stomach (n=43), brain (n=34), uterus (n=28), lung (n=22), kidney (n=13), skin (n=12), breast (n=11), bladder and urinary tracts (n=10), or other locations (n=29). The patient characteristics and HLA types for vaccination, are shown in Table I. These studies were undertaken at 10 different institutions (Kurume University Hospital, Kinki University Hospital, Okayama University Hospital, Nara Medical University Hospital, Hokkaido University Hospital, Niigata University Hospital, Kitasato

Table I. Severe adverse events observed in the clinical trials of the personalized peptide vaccination.

Disease	n	Median age years	SAE				
			Observed case no.	Event no.			
				Total	Grade 3	Grade 4	Grade 5
Prostate cancer	174	67.9	55	95	73	11	11
Colorectal cancer	74	58.5	5	6	1	1	4
Pancreatic cancer	51	64.8	20	81	65	3	13
Gastric cancer	43	58.7	1	1	0	0	1
Malignant brain tumor	34	49.6	2	2	1	1	0
Cervical cancer	28	49.9	3	5	5	0	0
Non-small cell lung cancer	23	60.5	2	2	1	0	1
Renal cell cancer	13	57.8	2	2	2	0	0
Melanoma	12	57.3	1	1	0	0	1
Breast cancer	11	54.3	3	4	3	0	1
Bladder cancer	8	66.6	5	6	1	3	2
Others	29	63.6	3	10	6	2	2
Total	500	61.8	102	215	158	21	36

University Hospital, Kansai Medical University Hirakata Hospital, Yamaguchi University Hospital, and Kyoundo Hospital in Japan), and were approved by the ethics review committee of each institution. The number of administered vaccinations varied widely, from 3 to 112 per patient, with the most prolonged vaccination periods being for the prostate cancer patients. Most of the safety, immune, as well as clinical responses in these studies have been previously reported (5-10,14-25). Studies are currently underway to obtain vaccination results for the treatment of pancreatic and breast cancer, as well as for the HLA-A3 supertype-positive patients. Results obtained after October 2008 have not been included in this study (unpublished data). The detailed patient characteristics of the 500 patients, including their immunological responses and clinical evaluations, are also currenlty being studied for the purpose of identifying biomarkers to predict clinical benefits (Noguchi *et al*, unpublished data).

Treatment regimens. Personalized peptide vaccination is based on a pre-vaccination measurement of the peptide-specific CTL precursors and anti-peptide IgG in the circulation of cancer patients, reactive to vaccine candidates, followed by the administration of only reactive peptides (up to 4 peptides) with Freund's incomplete adjuvant (ISA51; Seppic, Paris) as reported previously (5-10). A total of 78 candidate peptides (32 peptides for HLA-A24, 37 for -A2 and 8 for -A3 supertype-positive patients) were used in the personalized peptide vaccination (5-10). All of these peptides can induce the HLA-A24, A2- and -A3 supertype-restricted and tumor-specific CTL activity in the peripheral blood mononuclear cells (PBMCs) of cancer patients.

Physical examinations and baseline blood tests were repeated at 2-week intervals, and patients were questioned about adverse events, including their severity and frequency. The severity of adverse events was scored according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3 (2003). The SAEs were evaluated by the Institutional Safety Evaluation Committee (ISEC). Imaging studies to determine the extent of disease were performed at intervals of 3 months and repeated after 3 to 6 months to identify patients with responses. Patients were assigned a response category according to the Response Evaluation Criteria in Solid Tumors, the revised version of the WHO criteria published in the WHO Handbook for Reporting Results of Cancer Treatment, June 1999 (Final).

Results

SAEs. A total of 215 SAEs in 102 patients and their grades were recorded during the vaccination (Table I). There were 158 grade 3, 21 grade 4, and 36 grade 5 SAEs. The main causes for these events were cancer progression (152 SAEs in 78 patients), combined cancer treatments other than vaccination (35 SAEs in 21 patients), diseases other than cancer (20 SAEs in 19 patients), peptide vaccines (6 SAEs in 6 patients), and suicide (1 in 1 patient). The frequencies of SAEs were high in the bladder, pancreas and prostate cancer patients, whereas they were low in the gastric and colon cancer patients, and also in patients with malignant brain tumors.

The 6 vaccine-related SAEs, all grade 3, consisted of skin reactions at each injection site, cellulitis around the injection site, edemas of the head and neck regions, colitis, rectal bleeding and bladder-vaginal fistulae (Table II). Each of these cases is briefly described in the next section.

Case reports of the vaccine-related SAEs. Grade 2 inflammatory skin reactions at the injection sites (thigh regions)

Table II. Vaccine-related severe adverse events.

Case ID	Age at entry	Gender	Disease	Total no. of vaccinations	Onset of SAE (vaccination times)	SAE	CTCAE grade	Clinical outcomes		
								BCR	PFS	OS
K-GEM-005	73	F	Pancreatic cancer	77	48	Dermatology/ skin-other (cellulitis)	3	SD	803	1123
K-GEM-008	54	M	Pancreatic cancer	23	19	Injection site reaction-ulceration	3	SD	153	362
EBO-112P	77	M	Prostate cancer	104	102	Edema: Head and neck	3	PR	437	2430
EBL-002	61	M	NSCL	23	7	Colitis	3	SD	323	668
EBG-101	68	F	Cervical cancer	10	10	Hemorrhage, GI-rectum	3	PR	323	323
GY-II-004	75	F	Cervical cancer	29	25	Fistula, GU-bladder/ vagina	3	SD	789	804

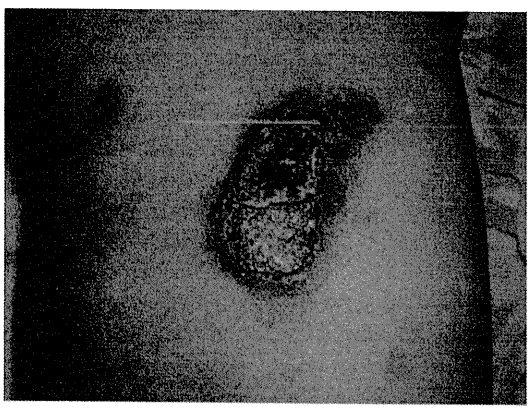

Figure 1. A skin ulcer at the injection site. Grade 3 ulcerations appeared at the previous injection sites of the thigh regions after the 19th vaccination in the abdominal region, in a patient with advanced pancreatic cancer (K-GEM-008).

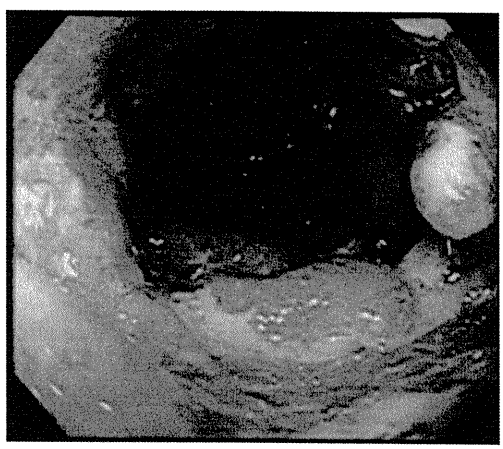

Figure 2. Colitis associated with ulcers. Examination with a sigmoid fiberscope revealed colitis associated with ulcers in a patient with advanced non-small cell lung cancer (EBL-002).

appeared after the 29th vaccination in a 73-year-old female patient with advanced pancreatic cancer (K-GEM-005, stage IVb), and therefore the vaccination interval was extended from 2 to 3 weeks in this patient (Table II). However, grade 3 cellulitis appeared at the injection site after the 48th vaccination in this patient, and consequently both the vaccination and gemcitabine were terminated for 4 weeks. After the disappearance of cellulitis, the vaccination and gemcitabine were resumed and continued until the 77th vaccination. The best clinical response (BCR) was stable disease (SD) with a progression free survival (PFS) of 803 days and an overall survival (OS) of 1123 days.

Grade 2 inflammatory skin reactions at the injection sites (the thigh regions) appeared after the 15th vaccination in a 54-year-old male patient with advanced pancreatic cancer (K-GEM-008, stage IVb), and consequently the injection sites were changed from the thigh to the side-abdominal regions (Table II). However, grade 3 ulcerations appeared at the previous injection sites in the thigh regions after the 19th vaccination. The clinical trial was terminated after the 23rd vaccination due to the skin ulcers in the thigh regions. The BCR was SD with a PFS of 186 days and an OS of 362 days. A representative ulcer at the injection site is shown in Fig. 1.

Grade 3 edema of the head and neck regions appeared 6 days after the 102nd vaccination in the subcutaneous thigh regions in a 77-year-old male patient with advanced hormone refractory prostate cancer (EBO-112P) who had been

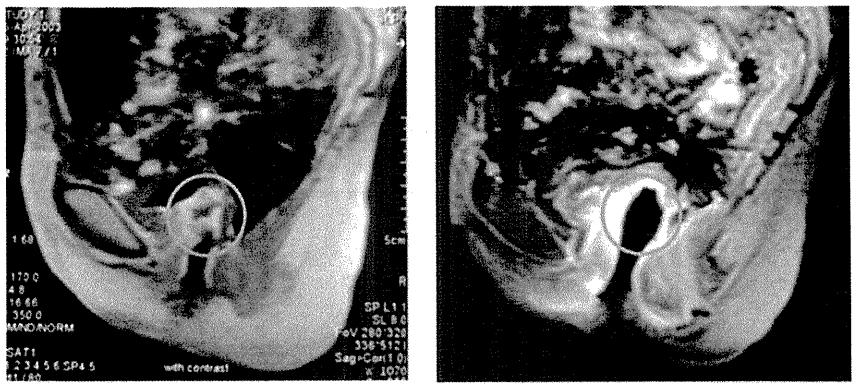

Figure 3. Bladder-vaginal fistula. Magnetic resonance imaging revealed the disappearance of the tumor mass after the 27th vaccination in a patient with advanced cervical cancer (GY-II-004).

responding well to the vaccination for a long period of time (Table II). The ISEC permitted the continuation of the vaccination therapy with careful observation, so the patient received the 103rd vaccination 14 days after the 102nd vaccination. Grade 3 edema of the head and neck region re-appeared 13 days after the 103rd vaccination. The patient was hospitalized for treatment, and the edema disappeared thereafter. The vaccination was terminated after the 104th vaccination based on the recommendations of the ISEC. The BCR was a partial response (PR) with a PFS of 437 days and an OS of 2430 days.

Grade 2 diarrhea appeared in a 61-year-old male patient with advanced non-small cell lung cancer (EBL-002, stage IVb), after the 4th vaccination (Table II). The diarrhea became more frequent after the 5th vaccination, and the vaccination interval was prolonged from 2 to 4 weeks. Examination with a sigmoid fiberscope revealed localized colitis. As the patient experienced no diarrhea thereafter, the interval was shortened again to 2 weeks after the 17th vaccination. Grade 3 diarrhea appeared after the 19th vaccination, and the vaccination interval was again prolonged from 2 to 4 weeks. However, the diarrhea and associated rectal bleeding continued. Examination with a sigmoid fiberscope revealed colitis associated with ulcers (Fig. 2). The patient was hospitalized for treatment, and the symptoms disappeared thereafter. The vaccination was terminated after the 23rd vaccination based on the recommendations of the ISEC. The BCR was SD with a PFS of 323 days and an OS of 668 days.

Constipation and rectal narrowing appeared after the 5th vaccination in a 68-year-old female patient with advanced cervical cancer (EBG-101, stage IV) who had a history of whole pelvic radiation therapy (60 Gy). A colostomy was carried out based on the diagnosis of radiation colitis. The patient re-entered the clinical trial. Grade 3 rectal bleeding with anemia appeared after the 7th vaccination, and blood transfusion was required in order to continue the treatment. Examination with a colon fiberscope revealed redness and swelling of the rectal mucosa, and a diagnosis of radiation colitis was made again. No invasion of cancer cells was observed. The ISEC concluded that the rectal bleeding was mainly caused by radiation colitis, and the vaccination therapy was considered not to have played a role. The dose of vaccination was reduced from 3 to 1 mg/peptide based on the recommendations of the ISEC. The rectal bleeding disappeared thereafter. The BCR was PR with an OS of 323 days. The patient died as a result of sepsis due to pyelonephritis, but not due to the progression of cancer.

Incontinence of urine appeared after the 24th vaccination in a 75-year-old female patient with advanced cervical cancer (GY-II-004, stage IV) who had a history of whole pelvic radiation therapy (60 Gy), and was diagnosed as a bladder-vaginal fistula. The tumor mass disappeared after the 27th vaccination (Fig. 3). The ISEC concluded that the fistula was mainly caused by vaccination-induced anti-tumor responses at the tumor sites, but the involvement of radiation colitis was not excluded. The vaccination was terminated after the 29th vaccination based on the recommendations of the ISEC. The BCR was SD with a PFS of 789 days and an OS of 806 days.

Immune responses and clinical responses at the onset of SAE. We next examined whether boosted immune responses were truly involved in the 6 cases of vaccine-related SAEs (Table II). Both CTL responses and IgG responses to each of the vaccinated peptides around the onset of SAEs, are shown in Table III. Both CTL and IgG responses to at least 2 peptides were observed in all patients. CTLs to all 4, 3, or 2 peptides were observed in 3, 1, or 2 patients in quadruplicate assays, respectively. All 4 out of 4 wells tested positive for 4 patients, while 3 out of 4 wells tested positive for 3 patients, indicating that the CTL precursor frequencies in post-vaccination PBMCs around the onset of the vaccine-related SAEs were much higher than those in the pre-vaccination PBMCs. Furthermore, the amounts of IFN-γ exceeded 500 ng/ml in most wells for all patients, suggesting the elevating activity of peptide-specific CTLs. Similarly, IgG responses to the vaccinated peptides were observed in 5 out of 6 patients. In addition, the IgG titers in post-vaccination plasma increased >100-fold in these 5 patients compared to those in pre-vaccination plasma. These results

Table III. Antigen-specific CTL and IgG responses to the vaccinated peptides at the time of SAE onset.

Case ID	Vaccinated peptides	IFN-γ production (pg/ml)[a]		NIgG (FIU)[b]	
		Pre-vaccination	SAE onset	Pre-vaccination	SAE onset
K-GEM-005	SART3-109	- (0)	- (0)	130	20,936
	Lck-486	- (0)	1419, 553 (2)	69	1,116
	PTHrp-102	- (0)	- (0)	113	14,500
	EZH2-291	- (0)	2266, 1075, 684, 381 (4)	10	29
K-GEM-008	SART3-109	- (0)	299 (1)	184	3,929
	Lck-486	- (0)	- (0)	62	161
	HER2/neu-553	47 (1)	553, 190, 133 (3)	20	24,555
	PTHrp-102	- (0)	- (0)	36	38
EBO-112P	SART3-309	359, 130 (2)	4076, 2691, 2102, 1324 (4)	10	23,960
	Lck-246	136, 100 (2)	2950, 2198, 1197 (3)	25	26,434
	UBE2V-43	- (0)	876 (1)	120	26,231
	UBE2V-85	- (0)	>5000, >5000 (2)	113	20,258
EBL-002	SART2-93	123 (1)	262, 190, 123, 96 (4)	<10	<10
	SART3-315	336 (1)	269 (1)	<10	<10
	Lck-208	100, 65 (2)	229, 118, 77, 52 (4)	<10	<10
	Lck-486	112 (1)	257, 123, 96 (3)	<10	<10
EBG-101	Lck-422	142 (1)	>5000, >5000, 905, 842 (4)	<10	<10
	MAP-432	130, 103, 41 (3)	>5000, 524 (2)	<10	<10
	UBE2V-43	- (0)	2597, 2477, 402 (3)	244	28,567
	Lck-246	- (0)	>5000, >5000, 227 (3)	196	20,273
GYII-004	SART2-93	- (0)	395, 145 (2)	10	25
	SART3-315	- (0)	785, 144 (2)	11	215
	SART3-109	77 (1)	192 (1)	248	29,511
	Lck-208	- (0)	- (0)	134	19,159

[a]Values of IFN-γ production (pg/ml) in the positive wells are indicated. Number of positive wells in the quadricate cultures is also shown in parenthesis. [b]FIU, fluoresence intensity unit.

indicate that both cellular and humoral responses specific to the vaccinated peptides were truly boosted at the onset of the vaccination-related SAEs. The clinical responses of these 6 patients were 2 PRs and 4 SDs (Table II).

Discussion

In the present study, with the exception of vaccine-related SAEs, the frequencies of SAEs were high in the bladder, pancreas and prostate cancer patients, and low in patients with gastric and colon cancer, or malignant brain tumors. This difference could mainly have been due to the nature of the cancers themselves. The OS of advanced bladder and pancreatic cancer patients at the time of entry to the vaccination trial was very short, ranging from 5 to 8 months, compared to that of patients with advanced gastric and colon cancer (22,23). The exception was prostate cancer, and the OS of advanced prostate cancer patients was relatively long, ranging from 12 to 17 months.

The main reason for the high frequency of SAEs in advanced prostate cancer could be the prolonged vaccination cycles. The median number of vaccinations for advanced prostate cancer patients was 16, with a range of 3 to 112 vaccinations, whereas the median number for patients with other types of advanced cancer was from 6 to 9, as previously reported (4-10,14-25).

Skin reactions at the injection sites were expected, as repeated vaccinations of the peptides along with ISA51 in the subcutaneous regions should elicit inflammatory responses (26), which in turn can result in SAEs in certain cases (4). In addition, anti-tumor responses at the cervical region in cervical cancer patients with a history of radiation therapy and thus are at risk of radiation colitis, could be a risk factor for vaccination-related SAEs.

The number of vaccinations in these 6 cases at the time of SAEs were relatively large, ranging from 7 to 102, as these patients were good responders, suggesting that the vaccination-related SAEs appeared more frequently in patients

who were considered to be good responders. This assumption could be supported by the fact that both cellular and humoral responses specific to the vaccinated peptides, were truly boosted around the onset of the vaccination-related SAEs in all 6 patients.

In conclusion, we show that the majority of SAEs occurring after peptide vaccination for advanced cancer patients were caused by cancer progression. However, it is recommended that physicians should be on guard for vaccine-related SAEs, despite their low incidence.

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BJUI
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A phase I study of personalized peptide vaccination for advanced urothelial carcinoma patients who failed treatment with methotrexate, vinblastine, adriamycin and cisplatin

Kazumasa Matsumoto, Masanori Noguchi*, Takefumi Satoh, Ken-Ichi Tabata, Tetsuo Fujita, Masatsugu Iwamura, Akira Yamada†, Nobukazu Komatsu‡, Shiro Baba and Kyogo Itoh‡

*Department of Urology, Kitasato University School of Medicine, *Clinical Research Division and †Cancer Vaccine Division of the Research Centre for Innovative Cancer Therapy, Kurume University School of Medicine, and ‡Department of Immunology, Kurume University School of Medicine, Kurume, Japan*

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Study Type – Therapy (case series)
Level of Evidence 4

What's known on the subject? and What does the study add?
This phase I study showed the safety and boosted immune responses of personalized peptide vaccination for advanced urotherial carcinoma.

This study showed feasibility of personalized peptide vaccination as a new therapeutic modality for advanced urotherial carcinoma patients who failed cisplatin-based chemotherapy.

OBJECTIVE

- To investigate the safety and immune responses of 12 consecutive weeks of once-weekly personalized peptide vaccine (PPV) administration in patients with advanced urothelial carcinoma (UC) for whom therapy with methotrexate, vinblastine, adriamycin and cisplatin (MVAC) has failed.

PATIENTS AND METHODS

- A phase I trial was designed. Ten patients with MVAC-refractory advanced or metastatic UC were treated with weekly personalized peptide vaccine 12 times using positive peptides chosen from 14 and 16 peptides in patients with human leucocyte antigens A24 and A2, respectively.
- Peptide-specific cytotoxic T lymphocyte precursor analysis by interferon-γ production and peptide-reactive immunoglobulin G (IgG) using an enzyme-linked immunosorbent assay was monitored during the treatment.

RESULTS

- The peptide vaccination was safe and well tolerated with no major adverse effects. Increased cytotoxic T lymphocyte response and the anti-peptide IgG titre were revealed by the post-vaccination sera in eight patients.
- Clinical responses were as follows: one complete response, one partial response, two stable disease and six progressive disease.
- Median progression-free survival and overall survival were 3.0 and 8.9 months, respectively. In the four responders, median progression-free survival and overall survival were 21 and 24 months, respectively.

CONCLUSIONS

- This phase I study showed the safety of and boosted immune responses in response to PPV for advanced UC.
- The potential efficacy of 12 consecutive weekly vaccinations with PPV in patients with advanced UC merits further investigation based on these findings.

KEYWORDS

urothelial carcinoma, bladder cancer, peptide vaccine, personalized therapy, phase I clinical trial

INTRODUCTION

The currently available standard chemotherapy for advanced or metastatic urothelial carcinoma (UC) is a cisplatin-based treatment that includes methotrexate, vinblastine, adriamycin and cisplatin (MVAC) or gemcitabine and cisplatin [1–4]. However, there are no established therapeutic modalities for patients with UC who fail with these cisplatin-based therapies. Therefore, new approaches should be taken, and one of them could be specific immunotherapy. Recent advances in tumour immunology have resulted in the identification of a number of antigens and their peptides that are recognized by tumour-reactive and human leucocyte antigen (HLA) class I-restricted

cytotoxic T lymphocytes (CTL) [5]. Cancer vaccines have emerged as a promising therapeutic approach [6]. The efficacy of intravesical BCG in the treatment of superficial disease suggests a role for developing immune recognition strategies to enhance the treatment of UC. The presence of tumour-infiltrating CD8 T cells has been associated with survival in patients with UC [7]. CD8-expressing T cells can also recognize the NY-ESO-1 antigen [8], which occurs in approximately 30–40% of muscle-invasive bladder cancer. A recent clinical trial found that all six of six patients developed antigen-specific immune responses when treated with NY-ESO-1 vaccine [9]. Additional work evaluating the impact of immunomodulating therapy is ongoing, including the use of the anti-cytotoxic T-lymphocyte antigen-4 antibody to overcome inhibitory signals down-regulating T cells [10]. However, their clinical responses have been limited. To overcome this limitation, we devised a new regimen of peptide-based vaccination that consists of measuring pre-existing CTL precursors and IgG reactive to many kinds of vaccine candidates, followed by administration of the positively reactive peptides (personalized peptide vaccination: PPV) [11–14]. A recently conducted randomized clinical trial of PPV for advanced prostate cancer patients showed a favourable clinical response in the vaccinated group [15], whereas most of the other randomized cancer vaccine trials failed to obtain better clinical responses in the vaccine group [16–18]. In this phase I study, we addressed the feasibility of PPV for patients with advanced UC for whom MVAC therapy had failed.

PATIENTS AND METHODS

Eligible patients were included if they were ≥18 years of age with HLA-A24 and/or HLA-A2 status, as determined by commercially available serological tests (SRL, Tokyo, Japan), and were measurable or assessable and histologically proven to have locally advanced (≥T3, N1) or metastatic (M1) UC that included the urinary bladder and upper urinary tract. All patients received surgical treatment or biopsy and MVAC therapy had failed. Previous chemotherapy with radiation therapy for local treatment of the primary lesion was allowed if completed at least 4 weeks before enrolment. Patients were eligible if their disease had progressed at any time after therapy for advanced or metastatic disease or within 12 months of neoadjuvant or adjuvant treatment. Patients were required to have an Eastern Cooperative Oncology Group performance status of 0 to 1, adequate bone marrow reserve (white blood cell count ≥3000/μL, lymphocyte count ≥1200/μL, platelets ≥75 000/μL and haemoglobin ≥10 g/dL), hepatic function (serum bilirubin ≤1.5 mg/dL), and renal function (serum creatinine ≤1.5 mg/dL), and an estimated life expectancy of at least 12 weeks. Patients with non-malignant systematic disease that precluded them from receiving therapy, including active infection, autoimmune disease, any clinically significant cardiac arrhythmia, or congestive heart failure were not eligible. Patients also had to be negative for hepatitis B and C antigens. Patients with CNS metastases, second primary malignant lesions, or clinically significant pleural effusions or ascites or who had used any investigational agent 1 month before enrolment were not eligible. The study protocol was approved by the institutional ethical review boards of Kitasato University and Kurume University, and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before entering this clinical trial.

The study design was for a non-randomized, open-label, phase I study in patients with advanced or metastatic UC previously treated with MVAC chemotherapy. The treatment was carried out at Kitasato University Hospital and Kurume University Hospital in the outpatients clinic. All immunological analyses were carried out at the Department of Immunology, Kurume University School of Medicine. The peptides used in the present study were prepared by Multiple Peptide Systems (San Diego, CA, USA) under the conditions of Good Manufacturing Practice. The peptide candidates consisted of $SART2_{93-101}$, $SART2_{161-169}$, $SART3_{109-118}$, $Lck_{208-216}$, $Lck_{486-494}$, $Lck_{488-497}$, $MRP3_{503-511}$, $MRP3_{1293-1302}$, $PAP_{213-221}$, $PSA_{248-257}$, $PSMA_{624-624}$, $EZH2_{735-743}$, $EGF\text{-}R_{800-809}$ and $PTH\text{-}rP_{102-111}$ for patients with HLA-A24, and $SART3_{302-310}$, $SART3_{309-317}$, $CypB_{129-138}$, $Lck_{246-254}$, $Lck_{422-430}$, $ppMAPkkk_{294-302}$, $ppMAPkkk_{432-440}$, $WHSC2_{103-111}$, $WHSC2_{141-149}$, $UBE2V_{43-51}$, $UBE2V_{85-93}$, $HNRPL_{140-148}$, $HNRPL_{501-510}$, $EZH2_{569-577}$, $PSCA_{21-30}$ and $EGFR_{479-488}$ for patients with HLA-A2 [8,9,13]. These peptides have the ability to induce HLA-A24-restricted or HLA-A2-restricted and tumour-specific CTL activity in peripheral blood mononuclear cells (PBMCs) of cancer patients, and are frequently expressed in various tumour cell lines [14,15,19]. The peptides were supplied in vials containing 3 mg/mL sterile solution for injection. Three milligrams of peptide with sterile saline was added in a 1:1 volume to the Monotide ISA-51 (Seppic, Paris, France), and then mixed in a Vortex mixer (Fisher, Alameda, CA, USA). The ISA51 is suitable for peptide vaccination because peptides solubilized in water phase are sequestered from peptidase-containing body fluid, and slow release of the peptides from the emulsion provides sustained antigenic stimulation [20]. The resulting emulsion (maximum of four peptides per vaccination) was injected subcutaneously into the femoral area, once a week for 12 weeks. This first cycle of treatment consisted of 12 consecutive weekly vaccinations. The cycle was repeated every 12 weeks for as long as the patients agreed to continue and their condition was considered appropriate for vaccination. Toxicity was evaluated in patients who received at least one vaccination, whereas both immunological and clinical evaluations were conducted in those who received more than six vaccinations. Blood samples for studies of immune responses were obtained on weeks 0, 6 and 12 during cycle 1. Supportive care could include blood transfusion and the administration of anti-emetics and analgesics, as appropriate. Further local therapy, including other chemotherapy regimens or radiation therapy, was allowed in patients with advanced disease after assessment of response to this regimen.

To measure peptide-specific CTL precursors, 30 mL peripheral blood was obtained before and after vaccination, and PBMCs were isolated by Ficoll–Conray density gradient centrifugation. Peptide-specific CTL precursors in PBMCs were detected using a previously reported culture method [21]. Briefly, PBMCs (1×10^5 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, Grand Island, NY, USA), 10% fetal calf serum, 100 U/mL interleukin-2 and 0.1 μM minimal essential medium non-essential amino acid solution (GIBCO BRL). Half of the medium was removed and replaced with a new medium containing a corresponding peptide (20 μM) every 3 days. After incubation for 14 days, these cells were harvested and tested for their ability to

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

TABLE 1
Patient characteristics

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-

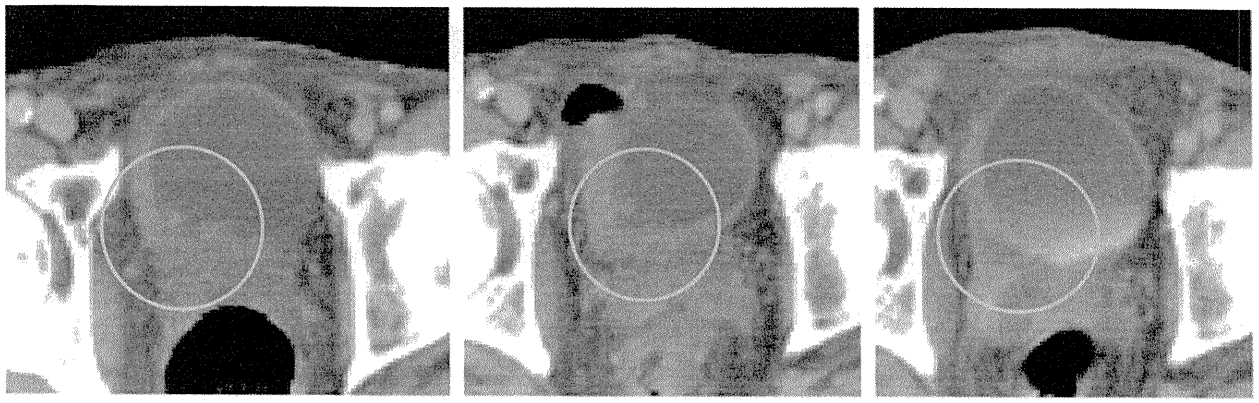
TABLE 2 Immune responses and clinical outcomes

Patient no. Clinical stage	Peptide	No. of vaccinations	Cellular response* Pre-	Cellular response* After 12th	Anti-peptide IgG† Pre-	Anti-peptide IgG† 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.*

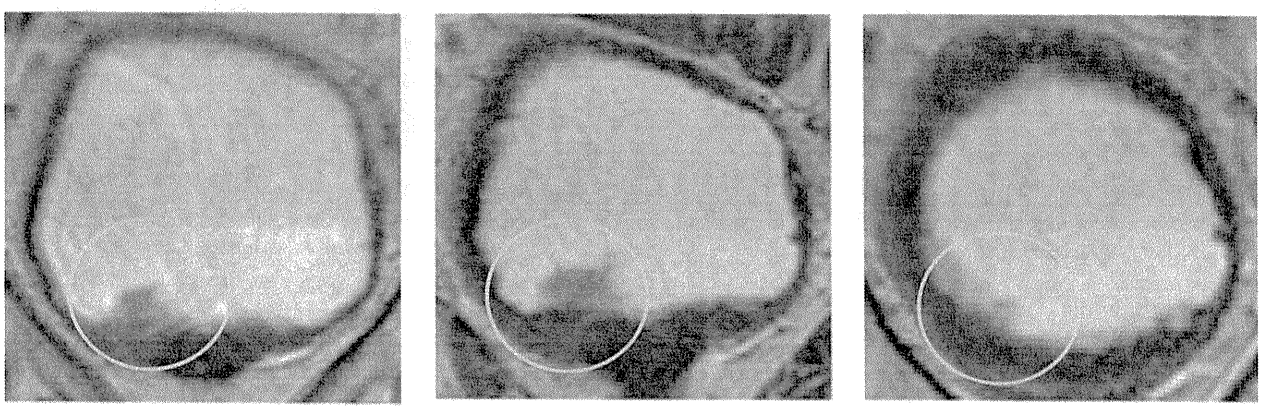
FIG. 1. The kinetic CT images of the tumour lesion of a patient with complete remission (A) and a patient with partial remission (B). The yellow circle indicates the tumour region. Left: pre-vaccination; middle: after the sixth vaccination; right: after the 12th vaccination. Cystoscopy findings of the 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.

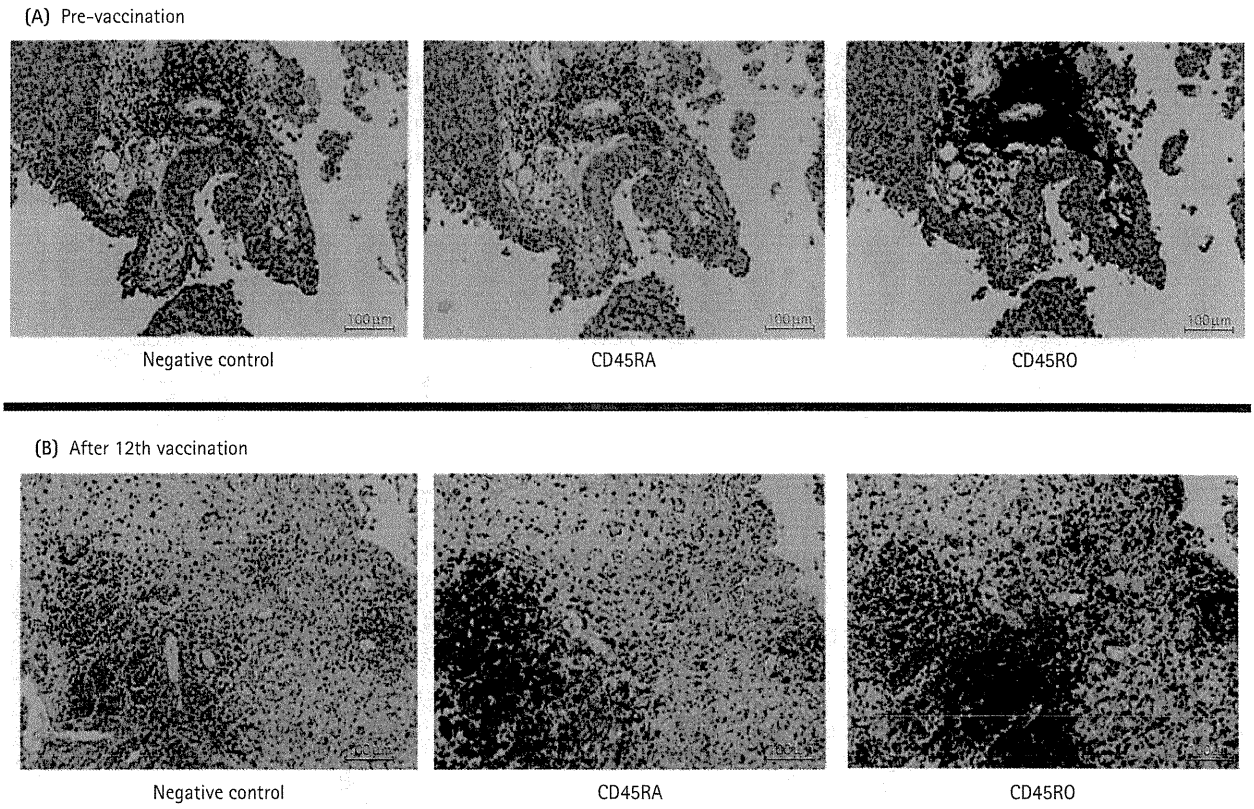
Representative non-haematological toxicity consisted of dermatological skin reactions including redness and heat at the vaccination site in all patients with grade 1 or 2 toxicity. There were no haematological toxicities or therapy-related deaths.

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

*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.*



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

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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Correspondence: Masanori Noguchi, Clinical Research Division of Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan.
e-mail: noguchi@med.kurume-u.ac.jp

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.

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

Masanori Noguchi,[1,2]* Fukuko Moriya,[2] Shigetaka Suekane,[2] Kei Matsuoka,[2] Gaku Arai,[3] Satoko Matsueda,[4] Tetsuro Sasada,[4] Akira Yamada,[5] and Kyogo Itoh[4]

[1]*Division of Clinical Research of the Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, Kurume, Japan*
[2]*Departments of Urology, Kurume University School of Medicine, Kurume, Japan*
[3]*Department of Urology, Dokkyo Medical University Koshigaya Hospital, Koshigaya, Japan*
[4]*Department of Immunology and Immunotherapy, Kurume University School of Medicine, Kurume, Japan*
[5]*Division of Cancer Vaccine of the Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, Kurume, Japan*

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* © 2011 Wiley-Liss, 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

*Correspondence to: Masanori Noguchi, MD, PhD, Department of Urology and Clinical Research Division of the Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan.
E-mail: noguchi@med.kurume-u.ac.jp
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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