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

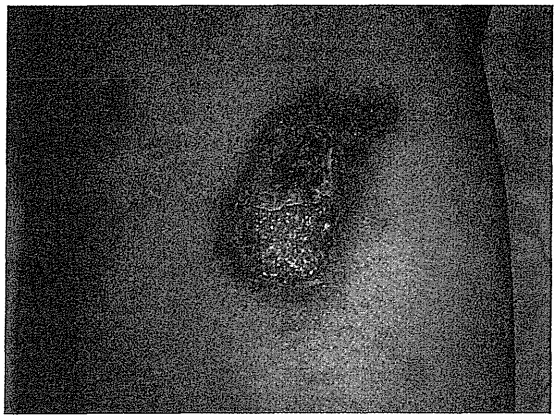

Figure 1. A skin ulcer at the injection site. Grade 3 ulcerations appeared at the previous injection sites of the thigh regions after the 19th vaccination in the abdominal region, in a patient with advanced pancreatic cancer (K-GEM-008).

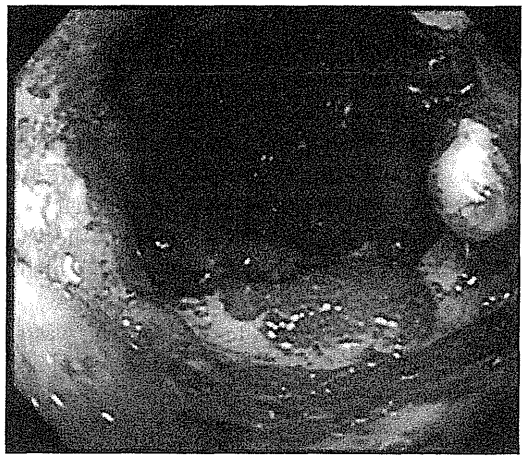

Figure 2. Colitis associated with ulcers. Examination with a sigmoid fiberscope revealed colitis associated with ulcers in a patient with advanced non-small cell lung cancer (EBL-002).

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

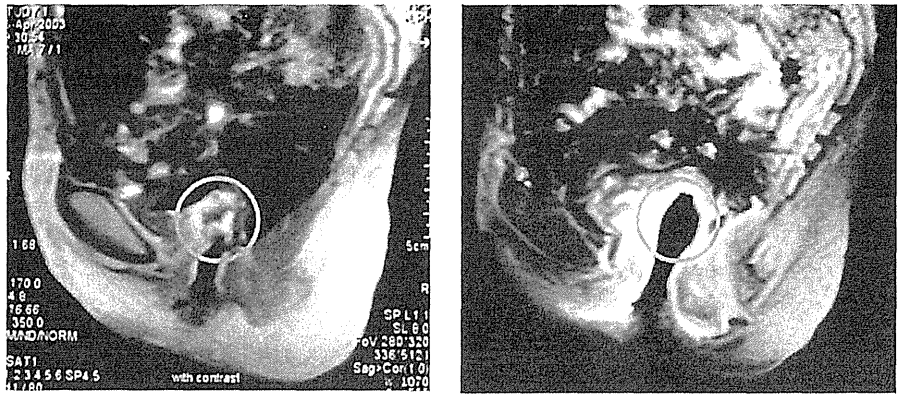

Figure 3. Bladder-vaginal fistula. Magnetic resonance imaging revealed the disappearance of the tumor mass after the 27th vaccination in a patient with advanced cervical cancer (GY-II-004).

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.

Acknowledgements

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A Phase I Study of Personalized Peptide Vaccination Using 14 Kinds of Vaccine in Combination With Low-Dose Estramustine in HLA-A24-Positive Patients With Castration-Resistant Prostate Cancer

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BACKGROUND. To evaluate the safety, tolerability, immune response, and antitumor activity of a combination of personalized peptide vaccination (PPV) and estramustine phosphate (EMP) in patients with castration-resistant prostate cancer (CRPC).
METHODS. In a phase I dose-escalation study, four peptides showing the highest levels of peptide-specific immunoglobulin G (IgG) to 14 vaccine candidates (ITK-1) were subcutaneously injected every week in three different dose settings (1, 3, and 5 mg per peptide) for 6 weeks with a low dose of EMP, and the patients were followed by maximum 2 years extension study either weekly or bi-weekly six times PPV as one course with a low dose of EMP.
RESULTS. Fifteen patients were enrolled in the phase I study. No serious treatment-related adverse events were observed. The most common adverse events were grade 2 skin reactions at the injection sites. The maximum acceptable dose of ITK-1 was 8.643 mg. There were no treatment-related systemic adverse events of grade 3 or more, and maximum tolerated dose could not be determined. Cytotoxic T lymphocyte responses measured by interferon-γ release assay were boosted in 10 of 15 (67%) patients, and IgG responses were boosted in 7 of 15 (47%) patients. Twelve patients proceeded to the extension study, and the median survival time was 23.8 months during a median follow-up of 23.8 months.
CONCLUSIONS. PPV treatment for HLA-A24 positive patients with CRPC could be recommended for further stages of clinical trials because of its safety and the higher frequency of boosting immune responses. *Prostate 71: 470–479, 2011.* © 2010 Wiley-Liss, Inc.

KEY WORDS: **personalized peptide vaccine; immunotherapy; phase I study; estramustine phosphate**

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INTRODUCTION

In the initial trials, peptide-based vaccine treatment of cancer patients rarely induced clinical responses and the levels of immune responses was low, indicating that the classical type of peptide vaccines did not have a promising future in the treatment of advanced cancer [1,2]. However, there have been slow but substantial advances in peptide vaccines and dendritic cell (DC)-based vaccines with regard to both clinical responses and immunological markers [3–12].

We previously reported that repeated multiple peptide vaccine regimen planned according to the pre-existing immunity (personalized peptide vaccine: PPV) could prolong the overall survival of patients with advanced cancer, and IgG specific to each peptide can frequently be detected in pre- and post-vaccination plasma [13]. In the previous trial, PPV was administered in 113 patients with advanced cancer, and the levels of peptide-specific cytotoxic T lymphocyte (CTL) precursors were measured by the interferon (IFN)-γ release assay and those of anti-peptide immunoglobulin (IgG) were estimated by enzyme-linked immunosorbent assay (ELISA). The level of anti-peptide IgG was a laboratory marker that predicted clinical responses to the PPV with a positive relationship to overall survival. Further, we showed that 58 patients with castration-resistant prostate cancer (CRPC) treated with a combination therapy of PPV and a low dose of estramustine phosphate (EMP) survived for a relatively long period of 17 months, which was comparable with the results of chemotherapy with docetaxel, and serious adverse events occurred less frequently in the study [4].

ITK-1 is a peptide set consisting of 14 kinds of peptide discovered as a HLA class I epitope, which being developed by Green Peptide Co., Ltd. All the 14 peptide candidates can induce CTLs, and each of them can induce HLA-A24-restricted and tumor-specific CTL activity in peripheral blood mononuclear cells (PBMCs) of cancer patients [14–18]. We have conducted a phase I study on PPV and low-dose EMP in HLA-A24-positive patients with CRPC in order to define the safety, tolerability, and immune and prostate-specific antigen (PSA) responses of this drug combination.

PATIENTS AND METHODS

Patients

This was a multi-center study and approved by each institutional review board (IRB) that evaluated it from the viewpoint of the science and ethics in all four hospitals in Japan before the initiation of the study. Patients who had a histological diagnosis of prostate adenocarcinoma (PC) and progressive disease (PD) by diagnostic imaging (computerized tomography; CT, magnetic resonance imaging; MRI or bone scintigraphy) or PSA after both androgen deprivation therapy either by castration or with luteinizing hormone-releasing hormone (LHRH) agonists and anti-androgen therapy, as well as oral EMP treatment were eligible. PSA progression was defined as at least three consecutive rises in serum PSA taken over 2 weeks apart, in the setting of castration levels of testosterone. Patients were required a washout period of at least 4 weeks before the first vaccination after the completion of prior hormone therapy, hormone-chemotherapy, chemotherapy, or immune therapy. Anti-androgen therapy was discontinued for at least 4 weeks before the first vaccination for patients receiving flutamide and 6 weeks for those receiving bicalutamide. All patients had an Eastern Cooperative Oncology Group performance status of 0 or 1, HLA-A24-positive type, and serum testosterone level $\leq$50 ng/dl, and were maintained on LHRH agonist therapy or castration. Adequate organ functions were required and were defined as white blood cell count $\geq$3,000/mm^3, lymphocyte count $\geq$1,200/mm^3, hemoglobin $\geq$9 g/dl, platelets $\geq$100,000/mm^3, total bilirubin $\leq$1.5 mg/dl, AST and ALT $\leq$2× (upper normal limit), and serum creatinine $\leq$1.4 mg/dl. Patients with comorbidities including serious cardiovascular, hepatic, nephritic, and hematological diseases $\geq$grade 3 of Common Terminology Criteria for Adverse Events (CTCAE), serious gastric ulcers, and infectious diseases with antibiotic treatment, were excluded. Radiation therapy or immunosuppressive treatment using a systematic steroid within the last 1 year was not permitted. All patients gave written informed consent approved by each IRB.

Study Design

This was a phase I open-labeled dose-escalation study. After a pre-vaccination measurement of peptide-specific IgG in the plasma of patients reactive to 14 kinds of vaccine candidate peptides (ITK-1) with the ability to induce CTLs, patients were treated with 6 weekly subcutaneous administration of the top four peptides showing the strongest antibody responses at three different dose settings (1, 3, and 5 mg/peptide), with daily oral EMP 313.4 mg in the phase I study. This was followed by a maximum of 2 years in an extension study of six PPVs either weekly or bi-weekly as one course. All patients were treated at the hospital during the first 1 week followed by outpatient clinic visits. ITK-1 consists of 14 kinds of peptides: $SART2_{93-101}$, $SART3_{109-118}$, $Lck_{208-216}$, $PAP_{213-221}$, $PSA_{248-257}$, $EGFR_{800-809}$, $MRP3_{503-511}$, $MRP3_{1293-1302}$, $SART2_{161-169}$,

$Lck_{486-494}$, $Lck_{488-497}$, $PSMA_{624-632}$, $EZH2_{735-743}$, and $PTHrP_{102-111}$. All peptides were prepared under Good Manufacturing Practice (GMP) compliance by American Peptide Company (San Diego, CA) and by PolyPeptide Laboratories (San Diego, CA), and were supplied in lyophilized vials; 4 mg, including inactive ingredients, under GMP compliance. Selected peptides were dissolved in 1 ml distilled water and emulsified with 1 ml of incomplete Freund's adjuvant (Montanide ISA-51VG; Seppic, Paris, France), under GMP compliance. Each of four peptides in 0.5 ml emulsion at a dose level of 1 mg/peptide (4 mg/2 ml), 1.5 ml emulsion at a dose level of 3 mg/peptide, and 2.5 mL emulsion at a dose level of 5 mg/peptide were injected subcutaneously into the thigh, the hip or the lower part of trunk area. Each peptide was independently injected nearby. EMP was administered orally as a 156.7 mg capsule, one capsule twice daily, for a total daily dose of 313.4 mg, half of the standard dose of EMP (626.8 mg/day) to avoid immunosuppression as reported in our previous study [19]. From the starting dose of 1 mg/peptide, subsequent dose levels were increased after the evaluation of the safety data by the Data and Safety Monitoring Committee (DSMC) according to the dose escalation design of the protocol. The initial cohort included six patients. If the DSMC recommended proceeding to the next level as a result of the safety evaluation of the prior level, new six patients were enrolled. The highest dose level enrolled three patients at first and was evaluated the safety data by the DSMC to include additional three patients. The maximum acceptable dose (MAD) was defined as the lowest dose level at which at least two-thirds of patients experienced grade 2 or greater injection site reactions after the sixth treatment. The maximum tolerated dose (MTD) was defined as the lowest dose level at which more than one-third of patients experienced grade 3 or greater systemic adverse events caused by ITK-1 after the sixth treatment. Adverse events were graded according to the CTCAE version 3.0 and were coded using MedDRA/J (Medical Dictionary for Regulatory Activities Terminology/Japanese) version 12.0. Patients who experienced no significant (≥CTCAE grade3) adverse events and no disease progression, and signed informed consent were eligible to extend treatment until disease progression or unacceptable adverse events occurred, or the patient met other withdrawal criteria.

Pretreatment and Follow-Up Studies

A complete history, physical examination, and routine laboratory studies, including complete blood counts, biochemical tests, ECG, relevant radiologic studies, PSA, and urinalysis were performed before treatment and repeated after every six injections.

Immune Responses

For evaluation of immune responses, peptide-specific CTL precursors in PBMCs and peptide-specific IgG levels in plasma were measured as described previously [13]. Also, peptide-specific IgG levels were measured using patient's plasma of the screening examination to select the best peptides. Briefly, 30 ml of peripheral blood samples were obtained from each patient to measure peptide specific CTL and IgG prior to vaccination, at the fourth and after the sixth vaccinations, and after every sixth vaccination in the extension study, and then the PBMCs and plasma were isolated by Ficoll–Conray density gradient centrifugation. We reported that the IgG specific to each peptide measured by Luminex system as the fluorescence intensity unit (FIU) could frequently be detected in pre- and post-vaccination plasma, and the level of peptide-specific IgG is a laboratory marker that predicts clinical responses to the PPV with a good relationship to overall survival [13,20]. Therefore, peptides were chosen on the basis of evaluation of peptide-specific IgG levels in plasma. 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 each peptide in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark) in 200 μl of culture medium. The culture medium consisted of 45% RPMI-1640 medium, 45% AIM-V® medium (Invitrogen Corp., Carlsbad, CA), 10% FCS, 20 U/ml of interleukin-2 (IL-2), and 0.1 mM MEM nonessential amino acid solution (Invitrogen Corp.), 36 mg/L gentamicin sulfate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) every 3 days for up to 12 days. On the 12th day of the culture, 24 hr after the last stimulation, these cells were harvested, washed three times, and then tested for their ability to produce IFN-γ in response to C1R-A2402 cells preloaded with either a corresponding peptide or HIV peptide (RYLRQQLLGI) as a negative control in HLA-A24. The target cells (C1R-A2402, 1×10^4/well) were pulsed with each peptide (10 μM) for 2 hr, and then effector cells (1×10^5/well) were added to each well with a final volume of 200 μl. After incubation for 18 hr, the supernatants (100 μl) were collected, and the amounts of IFN-γ were measured using an ELISA (limit of sensitivity: 10 pg/ml). All experiments were performed in quadruplicate assay.

Definition of Treatment Outcomes

Outcomes were assessed by post-therapy changes in serum PSA and immune responses. A post-therapy

TABLE I. Baseline Demographics

Characteristics	No. of patients (%)
No. of patients	15
Age, years	
Median	73
Range	63–78
ECOG PS	
0	14 (93)
1	1 (7)
Gleason score	
7	3 (20)
8	5 (33)
9	4 (27)
10	1 (7)
Unknown	2 (13)
PSA (ng/mL)	
Median	39.6
Range	0.2–354.4
Site(s) of metastasis	
None	4 (27)
Lymph node	2 (13)
Bone	6 (40)
Lymph node + bone	1 (7)
Other	2 (13)
Local therapy	
Prostatectomy	4 (27)
EBRT	3 (20)
No definitive local therapy	8 (53)
Hormone therapy	
Primary therapy only	1 (7)
≥2 therapies	14 (93)
Chemotherapy	
EMP	15 (100)
Other	2 (13)

ECOG PS, Eastern Cooperative Oncology Group performance status; PSA, prostate-specific antigen; EBRT, external-beam radiation therapy; EMP, estramustine phosphate.

decrease of PSA to a normal range was defined as a complete response (CR) and a decrease in PSA of ≥50% from baseline was defined as a partial response (PR) in the phase I study. Also, a post-therapy PSA decrease of <50% or an increase >25% from baseline were interpreted as no change (NC) [22] and PSA above 125% of the baseline PSA value was defined as PD. Positive immune responses were defined as post-IgG levels/pre-IgG levels ≥3, post-IFN-γ levels/pre- IFN-γ levels ≥3, respectively. All patients were followed up every 3 months for life. Data, except the survival data, were analyzed by November 2009 using SAS (Statistical Analysis System) software version 9.1.3. The Student's *t*-test and the chi-square test were used to compare quantitative and categorical variables, respectively. Overall survival was calculated from the study registration date to the date of the last follow-up or the death from any cause. The Kaplan–Meier method was used to estimate product-limit estimate curves with the survival data obtained in March 2010. Tests results were considered significant at a two-sided significance level of 5%. The analysis was performed by intent to treat.

RESULTS

Patient Characteristics

Fifteen patients were recruited to the study between April 2006 and September 2007. Patient characteristics are listed in Table I. All patients were HLA-A24-positive, and had hormone and EMP refractory prostate cancer. In addition, all 15 patients were evaluated for the safety and the efficacy of the PPV treatment.

Dose Escalation

The dose-escalation scheme is presented in Table II. Maximum dose escalation preplanned for each peptide of 5 mg/2.5 mL (4 peptides, 20 mg/10 mL) was achieved. There were no treatment-related grade 3 or 4 adverse events or deaths in this study. Grade 2 injection site reactions were observed in two of six patients in the first dose level of 1 mg/peptide, and five of six patients in the second dose level of 3 mg/peptide after the sixth treatment. At the 5 mg/peptide dose

TABLE II. The Results of Dose-Escalation in Phase I Study

Peptides dose level (mg/peptide)	No. of patients		No. of patients	
	Enroll	Discontinued or skipped[a]	MAD (≥grade 2 injection site reaction)	MTD (≥grade 3 systemic treatment-related AE)
1	6	0/6	2/6	0/6
3	6	0/6	5/6	0/6
5	3	3/3	3/3	0/3
Total	15	3/15	10/15	0/15

MAD, maximum acceptable dose; MTD, maximum tolerated dose; AE, adverse event.
[a]Patients were discontinued or skipped the treatment because both widespread grade 2 injection site reactions and patients' own requests.

level, three patients were treated, but the vaccination was skipped or discontinued in all three patients considering the ethical viewpoint because of patients' own requests and physical burden, caused by widespread grade 2 injection site reactions. After these treatment-related adverse events, two of three 5 mg/peptide dose level patients were entered in the extension study and then the dose level was reduced to 3 mg/peptide during treatment. The DSMC reviewed the results and recommended stopping the additional three enrollments for the dose level of 5 mg/peptide. Subsequently, the MAD for PPV was calculated to be 8.643 mg/4 peptide (2.161 mg/peptide) based on the logistic regression model.

Adverse Events

There were no treatment-related serious adverse events and no grade 3 or greater adverse events in the phase I study. In contrast, a grade 3 injection site reaction and a grade 3 pyrexia occurred in one patient each during the extension study. All treatment-related adverse events observed in whole study (phase I and extension study) are listed in Table III. The primary nonhematologic treatment-related adverse events were injection site reaction (93.3%), malaise (33.3%), edema peripheral (33.3%), and fatigue (20.0%). These adverse events were manageable with routine intervention. Hematologic adverse events were, grade 1 white blood cell count increased and grade 1–2 lymphocyte count decreased occurred in 4 of 15 (26.7%) and 3 of 15 (20.0%) patients, respectively. One patient at a dose level of 5 mg/peptide had a grade 1 blood fibrinogen increased, and another patient at a dose level of 3 mg/peptide had grade 1 blood triglycerides increased during the first course, and these changes returned to normal levels on the next course.

Immune Response

The best peptides for each patient were selected based on peptide-specific IgG levels for each peptide at the screening examination (data not shown). The results of the immune response in the first course are given in Table IV. After the sixth vaccination, IgG responses were increased in one of six patients with 1 mg/peptide, four of six patients with 3 mg/peptide, and two of three patients with 5 mg/peptide tested. CTL responses measured by IFN-γ release assay were increased in four of six patients with 1 mg/peptide, six of six patients with 3 mg/peptide, and zero of three patients with 5 mg/peptide tested.

Clinical Response

PSA response after the sixth vaccination was CR in one patient (6.7%) receiving 3 mg/peptide, PR in one patient (6.7%) receiving 1 mg/peptide, and PD in two patients (13.3%) receiving 5 mg/peptide. At the time of data analysis, nine patients had died and all deaths were attributed to prostate cancer or metastases. The median follow-up time for all patients was 23.8 months, ranging from 3.0 to 38.3 months. None of the patients was lost to follow-up during this analysis. The median overall survival was 23.8 months for all 15 patients (95% CI, lower limit was 15.6 months, upper limit was not estimated; Fig. 1).

DISCUSSION

We performed a multicenter, open-label, phase I trial to evaluate the safety, tolerability, immune response, and PSA response of a combination of escalating doses of PPV and low-dose EMP. All patients had hormone and EMP-refractory prostate cancer. The treatment regime was well tolerated at all dose levels, except the injection site reaction at the highest dose level of 5 mg/peptide observed in all three patients enrolled, and no MTD was established in this trial. The most common adverse event was injection site reaction. The concept of dose escalation in a phase I trial to identify an MTD may not be applicable to most therapeutic cancer vaccines [23]. Peptide vaccines based on non-mutated melanoma antigens such as MART-1/Melan A and gp100 were initially evaluated in a phase I setting, at doses ranging from 0.1 to 10 mg [24,25]. However, no toxicity was observed even at the highest doses, and in vitro analysis did not reveal any correlation between the peptide dose and the generation of specific T-cell reactivity from the PBMCs of the vaccinated patients. Neither the safety nor efficacy of the vaccine can be assessed in patients with a blunted immune response since both safety and efficacy depend on the immune response. In contrast, our initial trial for colorectal cancer patients with 0.3, 1, and 3 mg/injections of SART3 peptide showed that a dose of 3 mg/injection was better than that of 0.3 and 1 mg/injection based on the induction of cellular immune responses to both tumor cells and peptides [26]. The current phase I study also showed that a dose of 3 mg/injection was better than those of 1 and 5 mg/injection based on the induction of cellular immune responses to peptides, although total doses of four peptides were 4 mg/2 mL, 12 mg/6 mL, and 20 mg/10 mL. Under these conditions, there were no serious adverse events caused by ITK-1; however, grade 2 injection site reactions were observed in two of six patients receiving 1 mg/0.5 mL/peptide, five of six patients receiving 3 mg/1.5 mL/peptide, and three of three patients receiving 5 mg/2.5 mL/peptide in the phase I study. The vaccination was skipped or discontinued in three of three patients receiving 5 mg/2.5 mL/peptide

TABLE III. Treatment-Related Adverse Events for Castration-Resistant Prostate Cancer

	No. of patients experienced treatment-related adverse events during phase I study/whole study[a] by grade									Total (15 patients)	
	1 mg/peptide group (6 patients)			3 mg/peptide group (6 patients)			5 mg/peptide group (3 patients)			All grade	
MedDRA/J ver12.0 symptom: preferred Trem(PT)	G1 (PI/ Whole)	G2 (PI/ Whole)	G3 (PI/ Whole)	G1 (PI/ Whole)	G2 (PI/ Whole)	G3 (PI/ Whole)	G1 (PI/ Whole)	G2 (PI/ Whole)	G3 (PI/ Whole)	P I	Whole
Vomiting	1/1									1 (6.7%)	1 (6.7%)
Ventricular extrasystoles	0/1										1 (6.7%)
Fatigue	0/1	0/1		1/0	0/1					1 (6.7%)	3 (20.0%)
Injection site reaction	2/2	2/3		1/1	5/4	0/1		3/3		13 (86.7%)	14 (93.3%)
Malaise	1/2			0/1	0/1		0/1			1 (6.7%)	5 (33.3%)
Oedema peripheral	1/2	0/1			0/1		0/1			1 (6.7%)	5 (33.3%)
Pyrexia						0/1					1 (6.7%)
Aspartate aminotransferase increased	0/1										1 (6.7%)
Blood fibrinogen increased							1/1			1 (6.7%)	1 (6.7%)
Blood triglycerides increased				1/1						1 (6.7%)	1 (6.7%)
Crystal urine present	0/1										1 (6.7%)
Blood urine present				0/1							1 (6.7%)
Lymphocyte count decreased	1/1	1/1			1/1					3 (20.0%)	3 (20.0%)
Neutrophil count increased	0/1										1 (6.7%)
Urinary casts	0/1										1 (6.7%)
White blood cell count increased	0/1			1/2			1/1			2 (13.3%)	4 (26.7%)
White blood cells urine positive	0/1			0/1							2 (13.3%)
Bacteria urine identified				0/1							1 (6.7%)
Dizziness				0/1							1 (6.7%)
Dizziness postural				0/1							1 (6.7%)
Headache				1/0	0/1					1 (6.7%)	1 (6.7%)
Insomnia		0/1									1 (6.7%)
Cough	0/1										1 (6.7%)
Rash generalized					0/1						1 (6.7%)

[a]Whole study means phase I and extension study.

TABLE IV. Immunorogical Responses During the Personalized Peptide Vaccination

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)
1 mg	1	Lck-486	94	90	81	—	ND	ND	ND	—
		PSMA-624	<5	<5	<5	—	ND	ND	ND	—
		PTHrP-102	42	30	23	—	113	ND	ND	—
		SART3-109	31	24	21	—	ND	ND	ND	—
	2	Lck-486	310	206	976	Positive	667	ND	204	—
		MRP3-1293	38	21	28	—	ND	ND	186	Positive
		SART2-93	20	11	9	—	ND	ND	656	Positive
		SART3-109	27	13	18	—	899	ND	ND	—
	3	Lck-486	102	102	114	—	ND	78	ND	—
		Lck-488	45	46	52	—	462	ND	ND	—
		MRP3-1293	52	45	50	—	ND	ND	ND	—
		PAP-213	252	210	215	—	ND	ND	ND	—
	4	Lck-486	200	199	247	—	ND	ND	1,393	Positive
		Lck-488	<5	<5	<5	—	ND	ND	472	Positive
		PSA-248	117	99	109	—	ND	ND	ND	—
		PTHrP-102	171	138	142	—	564	ND	ND	—
	5	Lck-486	575	364	396	—	ND	117	57	—
		Lck-488	144	102	92	—	ND	ND	439	Positive
		MRP3-1293	91	64	51	—	133	160	ND	—
		PAP-213	90	70	77	—	3,764	ND	114	—
	6	MRP3-1293	779	586	411	—	ND	477	ND	—
		PSA-248	804	756	1,825	—	ND	ND	ND	—
		PTHrP-102	502	414	310	—	ND	93	753	Positive
		SART3-109	142	152	83	—	ND	ND	3,276	Positive
3 mg	7	Lck-486	202	216	9,028	Positive	ND	1,636	ND	—
		MRP3-1293	29	21	22	—	ND	ND	ND	—
		PAP-213	<5	<5	5	—	274	ND	1,494	Positive
		PSA-248	11	12	1,902	Positive	173	ND	ND	—
	8	Lck-486	298	261	287	—	2,543	ND	ND	—
		Lck-488	10	9	11	—	ND	ND	598	Positive
		MRP3-1293	23	21	23	—	ND	ND	ND	—
		PAP-213	8	5	9	—	ND	ND	2,613	Positive
	9	Lck-486	329	290	308	—	ND	ND	72	—
		Lck-488	128	103	106	—	ND	119	627	Positive
		MRP3-1293	53	36	40	—	ND	1,706	ND	—
		PAP-213	<5	<5	10,992	Positive	ND	683	ND	—

(*Continued*)

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.

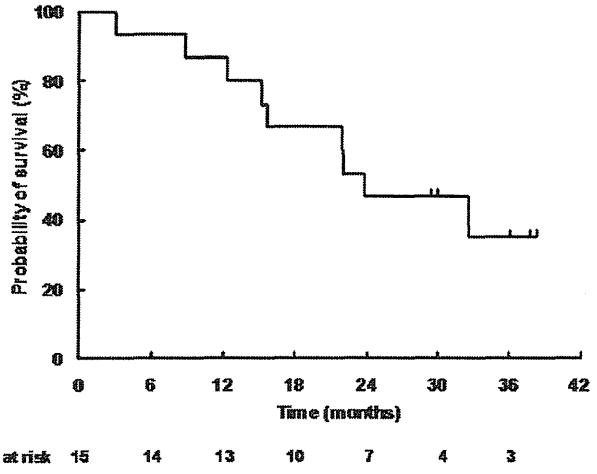

Fig. 1. Kaplan–Meier estimates of overall survival for 15 patients treated by personalized peptide vaccination with low-dose estramustine. Median overall survival is 23.8 months.

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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Assessment of immunological biomarkers in patients with advanced cancer treated by personalized peptide vaccination

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Key words: biomarker, personalized peptide vaccine, IgG, CTL, overall survival

Abbreviations: CTL, cytotoxic T lymphocytes; EBV, Epstein-Barr virus; ECOG, Eastern Cooperative Oncology Group; HLA, human leukocyte antigen; IFNγ, interferonγ; PBMC, peripheral blood mononuclear cells; HIV, human immunodeficiency virus; IgG, immunoglobulin G; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; PR, partial response; SD, stable disease; PD, progression disease; HR, hazard ratio; CI, confidence intervals; PSA, prostate-specific antigen

To investigate immunological biomarkers to predict overall survival of advanced cancer patients under treatment with personalized peptide vaccination, correlations between overall survival and biomarkers, including cytotoxic T lymphocyte (CTL) and immunoglobulin G (IgG) responses to the vaccinated peptides, were investigated in 500 advanced cancer patients who received a personalized peptide vaccination from October 2000–October 2008. The best clinical response was assessed for in 436 patients, 43 patients (10%) had partial response, 144 patients (33%) had stable disease and 249 patients (57%) had progressive, with a median overall survival of 9.9 months. Both lymphocyte counts prior to the vaccination ($p = 0.0095$) and increased IgG response ($p = 0.0116$) to the vaccinated peptides, along with performance status ($p < 0.0001$), well correlated with overall survival. To confirm the superiority of IgG response to CTL response, the samples from advanced castration-resistant prostate cancer patients who survived more than 900 days ($n = 20$) and those who died within 300 days ($n = 23$) were analyzed further. As a result, both the numbers of peptides, to which increased IgG responses were observed, and the fold increases in IgG levels were significantly higher in long-term survivors ($p = 0.000282$ and $p = 0.00045$). In contrast, CTL responses were not statistically different between the two groups. Both lymphocyte numbers and IgG response were thus suggested to be biomarkers of cancer vaccine for advanced cancer patients.

Introduction

The field of therapeutic cancer vaccines is currently in an active state of clinical investigations. There have been slow but substantial advances in peptide vaccines.[1-4] However, there are as yet no definite biomarkers to predict clinical responses, which hamper the development of cancer vaccines. Cytotoxic T lymphocyte (CTL) response has been reported as an immunological biomarker in many clinical trials, but the statistical powers have not been strong enough to warrant assignment as a definite biomarker.[4] This could be in part due to the lower sensitivity of CTL assays because of the CTL precursor frequency in the circulation is generally lower than 1 in 10,000 peripheral blood lymphocytes.[5,6] This could also be due to lower reproducibility as well as the requirement of in vitro incubation. More importantly, the clinical benefits of recent trials were not clear enough for the statistical analysis of biomarkers, which also made it difficult to find definite biomarkers.[1-4] Indeed, the majority of recently conducted randomized cancer vaccine trials failed to result in clinical benefits; in fact the clinical outcomes were worse in the vaccination groups than in the control groups.[7-9]

We previously reported that personalized peptide vaccination could prolong the overall survival of advanced cancer patients along with immunoglobulin G (IgG) responses as a biomarker in a relatively small number of patients.[10,11] In this study, we

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used the data from a total of 500 advanced cancer patients, who received personalized peptide vaccination conducted between October 2000 and October 2008, to investigate biomarkers that are predictive of their overall survival. Furthermore, we used samples from long-term survivors (more than 900 days of overall survival) and short-term survivors (less than 300 days of overall survival) with advanced castration-resistant prostate cancer (CRPC) under treatment with personalized peptide vaccination. It is well known that advanced CRPC patients rarely survive more than 2 years even if they receive global standard chemotherapy combined with hormone therapy.[12] Therefore, although only 43 patients were examined in subgroup analysis, the clinical benefits in the long-term survivors should be sufficiently large for the statistical analysis to identify definite biomarkers easily if any.

Results

Patient characteristics, immunological and clinical responses. The demographic, immunological responses and clinical characteristics of the 500 patients with advanced cancer are listed in **Table 1A and B**. The most frequent symptom of toxicity in the personalized peptide vaccination was a local skin reaction at injection sites. These symptoms were manageable through routine interventions as reported previously.[13-29] The best response to the personalized peptide vaccination was assessed in 436 patients. No complete responses (CR) were observed in either group. Forty-three patients (10%) had partial response (PR) and 144 patients (33%) had stable disease (SD). The remaining 249 patients (57%) had progressive disease (PD) without responses. Most of these clinical responses were already reported.[13-29] The response rate and disease control rate during the personalized peptide vaccination were 9.9 and 42.9%, respectively.

Correlation between overall survival and immune responses. The median follow-up for all 500 patients was 9.1 months (range, 1–105 months). Forty-five patients (9%) were alive at the end of the study (October 2009). Four hundred and forty-five patients died from advanced cancer and 10 patients died of other causes. The median overall survival time was 9.9 months with 1- and 3-year survival rates of 43 and 10.7%, respectively (**Fig. 1A**). Peptide-specific cellular and humoral immune activities were measured at 6-week intervals as long as patient samples were available. The total numbers of evaluable patients for CTL and IgG responses during the personalized peptide vaccination were 332 and 300, and positive results in CTL and IgG responses after the sixth vaccination were detected in 199 (60%) patients and in 187 (62%) patients, respectively. The median overall survival for patients with a positive IgG response was significantly longer than that for patients with a negative IgG response ($p = 0.0015$ by log-rank test; **Fig. 1C**), while an association between CTL response status and overall survival was not observed ($p = 0.167$ by log-rank test; **Fig. 1B**).

Analysis of predictors of overall survival. Cox proportional hazards regression analysis was performed to determine factors that are predictive of overall survival in the 500 patients listed above (**Table 2**). In univariate regression analysis, performance status ($p < 0.0001$), counts of lymphocytes ($p < 0.0001$), IgG response and age ($p = 0.002$) were found to be associated with survival. Gender, CTL response, HLA typing and vaccine interval were not significant factors. Forward stepwise multivariate analysis showed that only performance status ($p < 0.0001$; hazard risk 2.295; 95% CI, 1.653–3.188), counts of lymphocytes ($p = 0.0095$; hazard risk 1.472; 95% CI, 1.099–1.972) and IgG response ($p = 0.0116$; hazard risk 1.455; 95% CI, 1.087–1.948) were independent predictors of overall survival. None of the other variables were significant predictors of overall survival.

Comparison of immune responses between short- and long-term survivors. To statistically confirm the superiority of IgG response as a predictor to CTL response, samples from 20 patients who survived more than 900 days (long-term survivors) and those from 23 patients who died within 300 days (short-term survivors), among 174 patients with CRPC who received personalized peptide vaccination, were analyzed further. There were no statistical differences between the two groups with regard to clinical and pathological characteristics at the time of entry (**Table 3**). The only apparent difference was overall survival after the vaccination. Median survival times of long- and short-term survivors used for the analysis were 1,483 days and 189 days, respectively.

The frequencies of selection of each peptide candidate at the first vaccination between long- and short-term survivors were investigated to address if the peptides used were different between the two groups. There were no significant differences in the frequencies of selection of each peptide at the first vaccination between the two groups.

The levels of IgG reactive to each of the vaccinated peptides were measured for 21 of 23 short-term survivors and all 20 long-term survivors during both pre-vaccination and post-vaccination periods, and the representative results were given in **Table 4A and B**. The post-vaccination samples were not available from two short-term survivors. In short-term survivors, the numbers of peptides, against which a more than two-fold increase in IgG was observed, were 0 peptide in 10 patients, 1 peptide in 7 patients, 2 peptides in 3 patients and 3 peptides in 1 patient. In long-term survivors, numbers of peptides, to which increased IgG responses were observed, were 0 peptide in 3 patients, 1 peptide in 3 patients, 2 peptides in 5 patients, 3 peptides in 6 patients and 4 peptides in 3 patients ($p = 0.000282$). To better represent n-fold increase in IgG levels, the results were drawn in **Figure 2**, in which the vertical bars denote log 10 scores. In short-term survivors, the numbers of peptides, against which a more than 10-fold increase in IgG was observed, were 0 peptide in 16 patients, 1 peptide in 2 patients, 2 peptides in 2 patients and 3 peptides in 1 patient. In long-term survivors, the numbers of peptides, against which a more than 10-fold increase in IgG was observed, were 0 peptide in 5 patients, 1 peptide in 6 patients, 2 peptides in 5 patients and 3 peptides in 4 patients ($p = 0.00045$).

CTL activity against each of the vaccinated peptides was measured in 17 of 23 short-term survivors and all 20 long-term survivors during both pre-vaccination and post-vaccination periods (**Table 4A and B**). The post-vaccination peripheral blood mononuclear cells (PBMCs) needed for measurement of CTL responses were not available from 9 short-term survivors primarily because of rapid progression of cancer. In short-term survivors, the numbers

Table 1A. Characteristics, Immune responses and clinical responses of 500 patients with advanced cancer

Characteristics	Total		Groups of cancer											
			Prostatic cancer		Colorectal cancer		Pancreatic cancer		Gastric cancer		Brain tumor		Cervical cancer	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
No. of patients	500		174	35	74	15	50	10	42	8	33	7	28	6
Average Age, years	61.8		67.9		58.5		64.8		58.7		49.6		49.9	
Standard deviation	12.8		7.8		12.3		8.8		12.3		20.3		12.4	
Sex														
Male	353	71	174	100	52	70	32	64	29	69	18	55	-	
Female	147	29	-		22	30	18	36	13	31	15	45	28	100
Performance status (ECOG)														
0	333	67	144	83	47	64	33	66	20	48	7	21	16	57
1	118	24	25	14.5	23	31	16	32	16	38	6	19	9	32
2	31	6	1	0.5	4	5	0	0	6	14	8	24	3	11
3	18	3	4	2	0	0	1	2	0	0	12	36	0	0
Peptides bind for HLA														
A2	139	28	48	28	16	22	15	30	14	33	8	24	11	39
A24	332	66	109	63	58	78	31	62	28	67	25	76	17	61
A3-supretype	6	1	4	2	0	0	0	0	0	0	0	0	0	0
Mixed type	23	5	13	7	0	0	4	8	0	0	0	0	0	0
Average times of vaccination	14.7		17		13.9		16.3		9.8		13.1		14	
Standard deviation	15		18.9		11.8		14.3		9.8		11.2		9.9	
Treatment														
Vaccination alone	331	66	109	63	47	64	11	22	34	81	14	42	28	100
Combination	169	34	65	37	27	36	39	78	8	19	19	58	0	0
CTL response														
No. of evaluable case	332		111		60		40		25		26		20	
yes	199	60	75	68	32	53	26	65	15	60	17	65	13	65
no	133	40	36	32	28	47	14	35	10	40	9	35	7	35
IgG response														
No. of evaluable case	300		105		48		41		21		22		12	
yes	187	62	77	73	27	56	21	51	14	67	11	50	7	58
no	113	38	28	27	21	44	20	49	7	33	11	50	5	42
Best clinical response														
No. of evaluable case	436		155		68		41		35		30		23	
PR	43	10	29	19	1	1	4	10	0	0	5	16	3	13
SD	144	33	36	23	23	34	23	56	8	23	11	37	7	30
PD	249	57	90	58	44	65	14	34	27	77	14	47	13	57
Response rate (%)	9.9		18.7		1.5		9.8		-		16.7		13	
Disease control rate (%)	42.9		41.9		35.3		65.9		22.9		53.3		43.5	

Immunological responses were evaluated using the pre-and post-sixth vaccination samples.

of peptides, against which increased CTL responses were observed, were 0 peptide in 4 patients, 1 peptide in 6 patients and 2 peptides in 4 patients. In long-term survivors, the numbers of peptides, against which increased CTL responses were observed, were 0 peptide in 5 patients, 1 peptide in 12 patients, 2 peptides in 1 patient and 3 peptides in 2 patients ($p = 0.827009$).

Discussion

This study showed that both lymphocyte counts prior to the vaccination and increased IgG response to the vaccinated peptides, along with performance status, well correlated with overall survival of advanced cancer patients who received personalized peptide vaccination. Lymphocyte counts prior to vaccination shall be a biomarker primarily because lymphocytes are absolutely required for vaccine-mediated immune boosting. In addition, lymphopenia is recently reported to be an independent prognostic factor for overall survival in advanced cancers.[34] In contrast to lymphocyte counts, one might question why IgG response, but not CTL response, is a biomarker of the effectiveness of the peptide vaccination given that the vaccination primarily activates peptide-specific CTLs, but not B cells. We also brought up the same question when reporting on IgG responses as a biomarker following an investigation of 211 patients under treatment with personalized peptide vaccination.[10] Therefore, we extended that study in the present work and report convincing results showing that IgG response is superior to

Table 1B. Characteristics, Immune responses and clinical responses of 500 patients with advanced cancer

Characteristics	Groups of cancer											
	NSCLC		RCC		Melanoma		Brest cancer		Urotherial cancer		Others	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
No. of patients	22	4	13	3	12	2	11	2	10	2	31	6
Average Age, years	60.5		57.8		57.3		54.3		66.6		63.6	
Standard deviation	12.4		11.2		18.2		11.4		10.7		11.9	
Sex												
Male	11	50	11	85	7	58	0	0	9	90	16	52
Female	11	50	2	15	5	42	11	100	1,	10	15	48
Performance status (ECOG)												
0	14	64	10	77	7	58	5	46	6	60	16	52
1	5	23	2	15	3	25	4	36	3	30	8	26
2	3	13	1	8	2	17	1	9	1	10	7	22
3	0	0	0	0			1	9	0	0	0	0
Peptides bind for HLA												
A2	4	18	3	23	4	33	4	36	4	40	8	26
A24	18	82	9	69	8	67	7	64	6	60	16	52
A3-supretype	0	0	0	0	0	0	0	0	0	0	2	6
Mixed type	0	0	1	8	0	0	0	0	0	0	5	16
Average times of vaccination	13.8		23.5		12.3		9		11.9		10.8	
Standard deviation	15.4		15		6.6		9.8		6		13.4	
Treatment												
Vaccination alone	22	100	12	92	12	100	4	36	9	90	29	94
Combination	0	0	1	8	0	0	7	64	1	10	2	6
CTL response												
No. of evaluable case	11		10		8		6		3		12	
yes	6	55	2	20	6	75	1	17	2	67	4	33
no	5	45	8	80	2	25	5	83	1	33	8	67
IgG response												
No. of evaluable case	12		9		7		4		3		16	
yes	7	58	5	56	5	71	4	100	2	67	7	44
no	5	42	4	44	2	29	0	0	1	33	9	56
Best clinical response												
No. of evaluable case	21		12		11		10		7		23	
PR	0	0	0	0	0	0	0	0	1	14	0	0
SD	11	52	9	75	5	45	1	10	2	29	8	35
PD	10	48	3	25	6	55	9	90	4	57	15	65
Response rate (%)	-		-		-		-		14.3		-	
Disease control rate (%)	52.4		75		45.5		10		42.9		34.8	

Immunological responses were evaluated using the pre-and post-sixth vaccination samples.

CTL response in predicting the overall survival of advanced cancer patients under treatment with personalized peptide vaccination.

It is obvious that cellular immune responses shall be an important marker if appropriate assay conditions are defined and used. However, the current available T cell assays possess insufficient sensitivity and reproducibility for monitoring immune responses in vaccinated patients. Various T cell assays for quantifying and characterizing antigen-specific T cell responses, including ELISPOT, ELISA, intracellular cytokine staining (ICS), ^{51}Cr-release cytotoxicity assay, peptide-MHC multimer and proliferation assay (^{3}H-thymidine uptake and CFSE), have been extensively studied.[4,30,31] Using these T cell assays, increasing numbers of studies have reported significant correlations between clinical benefits and immunological responses in a limited number of patients.[4,30,31] However they are often inconsistent and unreproducible in other studies, because no universal standards have been established in the current T cell assays, which continue to be modified on a regular basis.[4,30,31] In fact, we have already tried several T cell assays, including delayed type hypersensitivity test and cytotoxicity assay, in our vaccinated patients, but their results were no better than the CTL precursor assay that we employed in the current study.[10] We also employed ELISPOT assay with the similar results (Noguchi M, et al., unpublished results). Therefore, we think that optimization and

standardization of T cell assay protocols, including the analysis, interpretation and reporting of data, may be crucial for future development of immune monitoring in cancer patients.[4,30,31] Nevertheless, it should be also noted that T cell assays have their inherent limitations. Even if innovated technologies are introduced and assay protocols are sophisticated, it will be difficult to dramatically improve their performance characteristics, such as sensitivity and reproducibility, because the frequencies of antigen-specific T cells are usually quite low even after vaccination.[5,6]

One might have several questions with regard to relationship between peptide-specific CTL responses and peptide-specific IgG responses, but we found no statistically significant correlation between the increased IgG responses and the increased CTL responses in 300 patients shown in **Table 1A and B** as well as 43 patients shown in **Table 4A and B**. We previously reported that both IgG and CTL responses were augmented in the samples after 6th vaccination from the majority of patients who showed PR responses.[19,23,25] We also demonstrated that there were no significant differences in overall survival between patients showing both CTL and IgG responses and those showing only IgG response.[10,11] These results suggest that boosted CTL responses are involved in tumor reduction, but not necessarily involved in prolonged overall survival.

We investigated the correlation between pre-vaccination lymphocyte counts and the induction of IgG responses in the 43 patients listed in **Table 4A and B**. As a result, there was no significant correlation between them. In addition, we addressed if boosted IgG responses to the vaccinated peptides were associated with concomitant increase of peptide-specific IgG to non-vaccinated peptides in the patients showing longer survivals shown in **Table 4A and B**. As a result, no such concomitant increase was observed in the majority of long survivors as well as short survivors listed in **Table 4A and B**. These results suggest that the boosting effect was really limited to the vaccinated peptides.

There could be several possible explanations for these unexpected results. Firstly, to the best of our knowledge, none of the previously reported studies involved more than several hundred cases under a single concept (personalization of peptide selection) of therapeutic peptide vaccination for advanced cancer patients. Although some of the clinical trials of peptide vaccination identified CTL response as a biomarker that predicts overall survival,[2,4,10,30] the numbers of patients were too small to obtain significant results. Furthermore, the clinical benefits of those peptide vaccination trials were not sufficiently large to enter randomized phase III trials. A number of poorly validated or controversial markers made it difficult to obtain approval of cancer vaccines as drugs. Indeed, there are no prospectively defined markers validated in large phase II or III studies at the time of writing.[7-9] Therefore, IgG response, but not CTL response, to the vaccinated peptides or proteins has the possibility to become a true biomarker that is predictive of the overall survival of cancer patients under treatment with cancer vaccine. In line with our observations, other researchers have also recognized the significance of B cell responses induced by vaccination with tumor antigens. Secondly, we previously reported that the personalized peptide vaccination mainly induced infiltration of CD45RO$^+$ T cells, but not that of CD8$^+$ T cells or CD20$^+$ B cells.[31] The results suggest that personalized peptide vaccination initially induced CD45RO$^+$ memory helper T cells to infiltrate into tumor sites, which in turn facilitated the proliferation of CD8$^+$ CTLs and B cells. Consequently, the activated CTLs eliminated cancer cells, while the activated B cells differentiated into plasma cells, which in turn produced IgG specific to the vaccinated peptides. Although the precise mechanisms, in which helper CD4$^+$ T cells are activated after vaccination with HLA class I-restricted

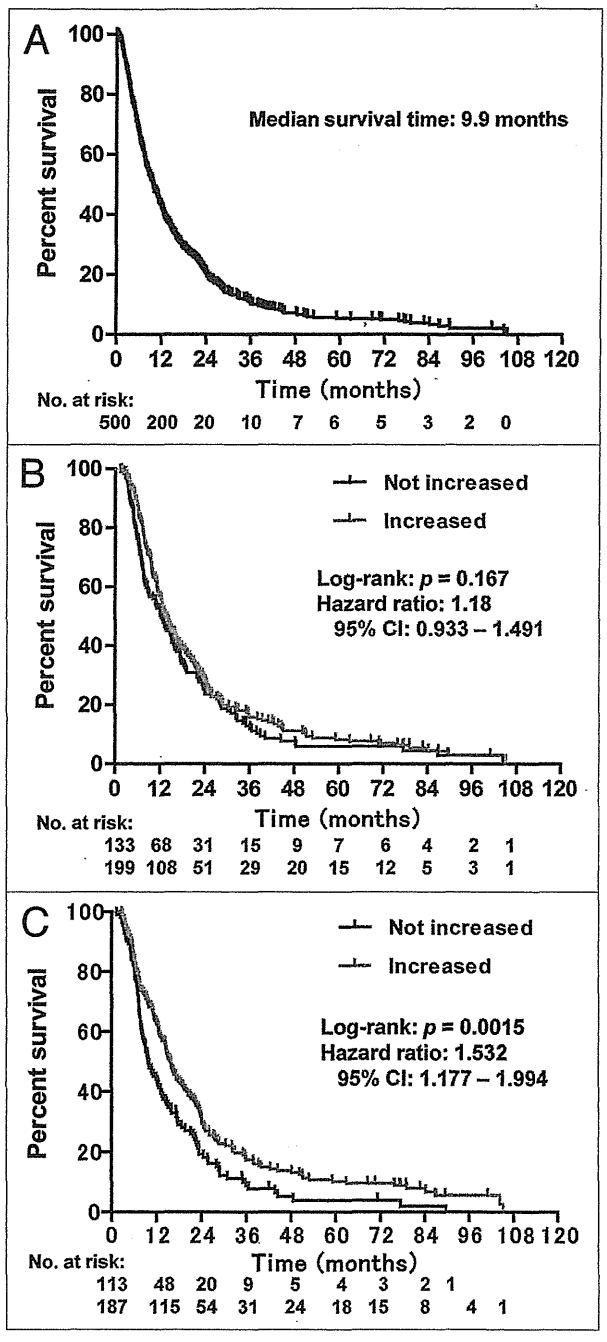

Figure 1. (A) Overall survival in 500 patients with advanced cancer treated by personalized peptide vaccination. Overall survival curves according to peptide-specific cellular (B) and humoral (C) immune response status.

Table 2. Univariate and multivariate analysis for overall survival using Cox regression models

	Univariate			Multivariate		
Factor	**p**	**HR**	**95% CI**	**p**	**HR**	**95% CI**
Performance status (ECOG) ≥ v < 1	<0.0001	2.4560	1.990–3.030	<0.0001	2.2950	1.653–3.188
Counts of lymphocytes < v ≥ 1,500/μL	<0.0001	1.6810	1.362–2.074	0.0095	1.4720	1.099–1.972
IgG responses no v yes	0.0015	1.4970	1.167–1.919	0.0116	1.4550	1.087–1.948
Age < v ≥ 63	0.0020	1.3420	1.113–1.617	-	-	-
Gender Male v Female	0.0984	0.8420	0.686–1.033	-	-	-
CTL responses no v yes	0.1587	1.1800	0.937–1.486	-	-	-
HLA typing A24 v others	0.2504	0.8900	0.729–1.086	-	-	-
Vaccine interval 1 week v ≥ 2 weeks	0.2117	0.8760	0.712–1.078	-	-	-

Lymphocyte and patient age are based on median values, and the remaining are treated as dichotomous variables.

Table 3. Baceline patient characteristics

	Long survivors		Short survivors		
	No	**%**	**No**	**%**	**p**
No. of patients	20		23		
Age, years					
Median	71		64		0.152
Range	54–78		50–80		
ECOG performance status					
0	20	100	20	87	0.236
1			3	13	
HLA typing					
A24	10	50	13	57	0.761
A2	8	40	8	35	
A24 and A2	2	10	2	8	
PSA, ng/ml					
Median	34.5		83		0.404
Range	2–330		2–296		
Gleason score					
7	6	30	3	13	0.299
8	9	45	10	43.5	
9	5	25	10	43.5	
Site of metastasis					
No	3	15	2	9	0.651
Bone only	14	70	17	74	
Bone and node	2	10	3	13	
Node/organ	1	5	1	4	
Progression free survival time, days					
Median	57		43		0.042
Range	14–926		14–96		
Survival time, days					
Median	1483		189		<0.0001
Range	699–2811		79–297		

peptides, still remain to be clarified, one possibility is that the peptides employed in this study may be presented not only in HLA class I but also in HLA class II and recognized by both CD8 and CD4 T cells, as has been reported in the PSA peptide at position 248–257 in prostate patients by our group and also in the Melan A 26–35 (A27L) peptide in melanoma patients.[32,33] Alternatively, the peptides employed in this study may be recognized by $CD4^+$ T cells on HLA class I molecules without requirement of CD8