Table 4B. Comparison of immune responses between short- and long-term survivors (continued)

	WHSC-141	0	0	negative	<10	<10	negative
34	SART3-315	0	0	negative	NT	NT	NA
	PSA-248	0	0	negative	29	4413	≥10
	PSM-624	0	0	negative	<10	<10	negative
	PAP-213	0	0	negative	41	25783	≥10
35	SART2-93	51	912	≥2	99	934	≥2
	Lck-488	108	61	negative	74	721	≥2
	PSA-152	0	0	negative	10	900	≥10
	PSA-248	0	0	negative	717	1058	negative
36	SART3-109	0	0	negative	184	228	negative
	Lck-208	56	0	negative	23	28	negative
	PAP-213	57	1802	≥10	13	379	≥10
	SART3-315	158	1121	≥2	NT	NT	NA
37	SART3-302	0	1417	≥10	40	11118	≥10
	SART3-309	0	0	negative	108	424	≥2
	PSA-170	0	0	negative	21	1221	≥10
	PSA-178	0	0	negative	32	1889	≥10
38	SART3-302	0	0	negative	309	15523	≥10
	CypB-129	0	0	negative	91	858	≥2
	PSMA-441	0	1163	≥10	NT	NT	NA
	PSMA-711	0	0	negative	NT	NT	NA
39	SART3-109	0	282	≥10	134	9562	≥10
	Lck-486	449	126	negative	14	12	negative
	PSA-248	157	172	negative	12	14507	≥10
	PTHrP-102	209	119	negative	16	11256	≥10
40	SART2-161	81	0	negative	1433	1451	negative
	SART3-109	0	0	negative	5368	24796	≥2
	PSA-248	0	0	negative	47	3854	≥10
	EZH2-291	0	784	≥10	2027	6674	≥2
41	SRAT3-109	0	0	negative	170	992	≥2
	Lck-488	0	0	negative	54	30278	≥10
	MRP3-1293	312	0	negative	21	3996	≥10
	PSA-248	78	0	negative	25	29669	≥10
42	CypB-129	464	0	negative	348	468	negative
	HNRL-501	0	436	≥10	859	1298	negative
	EIF-51	0	102	≥10	714	6797	≥2
	EZH2-569	0	899	≥10	2501	305	negative
43	CypB-129	140	0	negative	26	38	negative
	UBE-43	141	3424	≥10	27	1910	≥10
	EZH2-569	313	417	negative	18	446	≥10
	Her2-484	69	0	negative	<10	15	≥10

NA, not available; NT, not tested. [a]Values indicate IFN-γ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). 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 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. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 10.

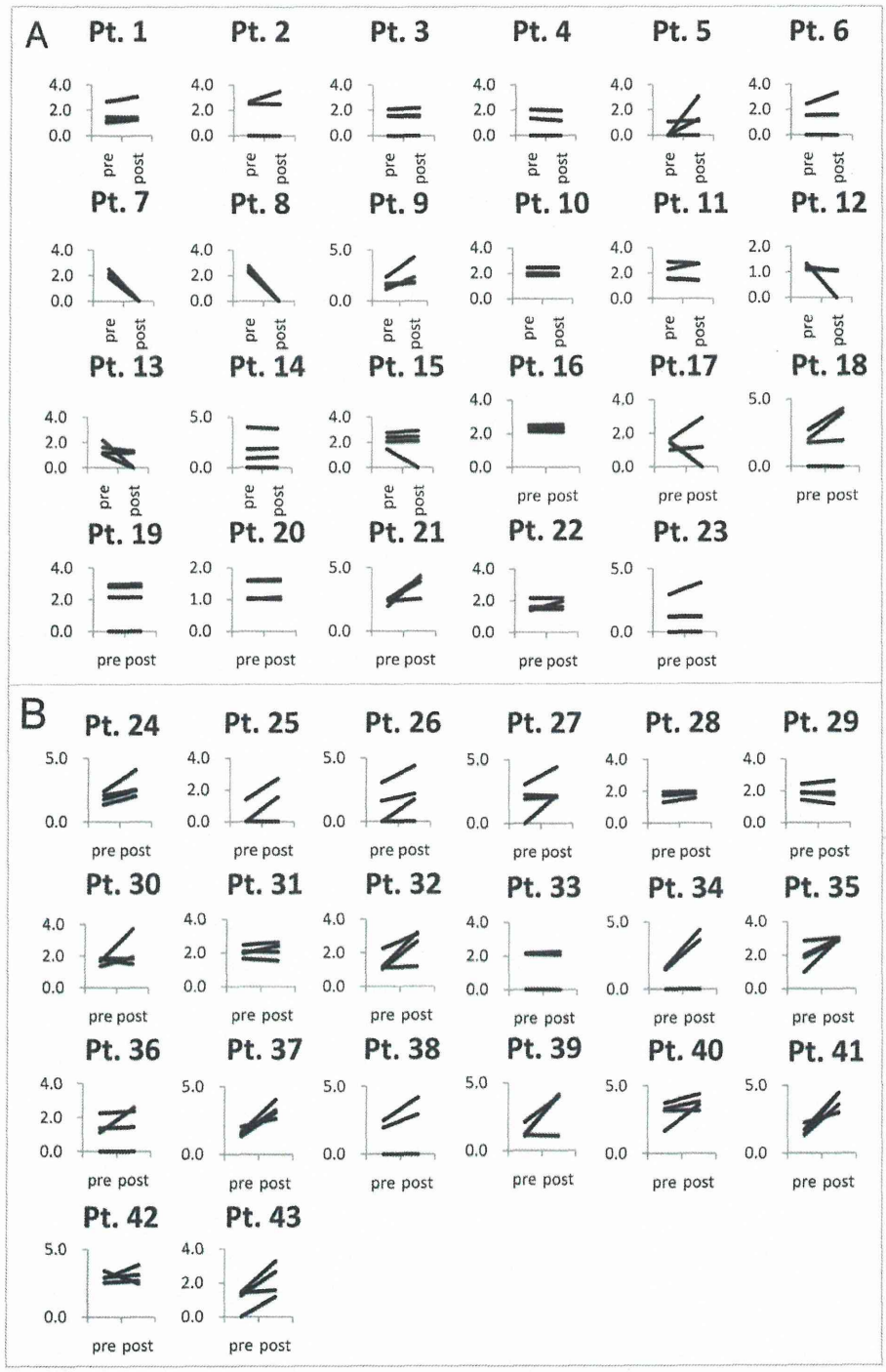

Figure 2. Changes of IgG levels reactive to each of the vaccinated peptides during pre- and post-vaccination periods (sixth) for short-term survivors (A) and long-term survivors (B). The vertical bars denote log 10 scores in order to better represent n-fold increases in IgG levels. NA, not available.

encoding tumor associated antigens by means of cDNA expression cloning technique reported by Boon et al.[40] Among many peptide candidates coded by these antigens, the peptides capable of inducing CTL reactive to tumor cells in HLA-class IA-restricted and peptide-specific manners were screened by incubation of PBMCs from cancer patients. Interestingly, many of these identified peptides were also recognized by pre-vaccination plasma IgG of cancer patients as reported previously.[41] Subsequently, to save limited source of patients' PBMCs, a large numbers of peptide candidates holding the motifs for binding to HLA-class IA molecules were at first tested for their ability to react to pre-vaccination patients' IgG, followed by testing their ability to induce HLA-class IA-restricted and peptide-specific CTL reactive to tumor cells in patients' PBMCs. Therefore, the peptides employed in this study mainly selected by their ability to be recognized by both cellular and humoral immunity. As far as we know, no other clinical trials of peptide-based cancer vaccine provided such peptides; other groups used the peptides capable of inducing only CTL without paying attention to their reactivity to IgG.

In conclusion, we have shown that IgG response is superior to CTL response as an immunological biomarker that is predictive of the overall survival of advanced cancer patients under treatment with personalized peptide vaccination. These results might provide new insights to better understand biomarkers of cancer vaccine for advanced cancer patients. Application of these results for the other types of cancer vaccine using common proteins or common peptides in a non-personalized manner could be worthy to consider.

Patients and Methods

Study population. This study was conducted through the serial collection of blood samples from 500

consecutive patients positive for HLA-A24, -A2 or -A3 supertypes with advanced cancer, who entered into phase I, I/II and II clinical trials for personalized peptide vaccination at 8 institutions (Kurume University Hospital, Kinki University Hospital, Okayama University Hospital, Hokkaido University Hospital, Niigata University Hospital, Kitasato University Hospital, Kansai Medical University Hospital and Yamaguchi University Hospital, Japan) between October 2000 and October 2008. The ethics review committee of each institution accepted the present project and blood samples were collected at baseline (before vaccination), at sixth vaccination, and during the follow-up period after written informed consent was obtained. All 500 patients suffered from advanced cancer originating in the prostate (n = 174), colon and rectum (n = 74), pancreas (n = 50), stomach (n = 42), brain (n = 33), uterus (n = 28), lung (n = 22), kidney (n = 13), skin (n = 12), breast (n = 11), bladder and urinary tracts (n = 10) and elsewhere (n = 31) (**Table 1A and B**). The safety, immune responses and clinical responses in most of those studied had been reported previously.[6,13-29] The exceptions were the results of vaccinations against bladder cancer, breast cancer, some pancreatic cancer cases, and those from HLA-A3 supertype-positive patients. These unpublished results have now been submitted for publication or are under preparation based on results obtained after October 2008. In the sub-analysis, 20 patients who survived more than 900 days (long-term survivors) and 23 patients who died within 300 days (short-term survivors) were selected to compare immune responses from a total of 174 patients with CRPC.

Personalized peptide vaccination and immunological assessment. Personalized peptide vaccination is based on a pre-vaccination measurement of peptide-specific CTL precursors and anti-peptide IgG in the circulation of cancer patients reactive to vaccine candidates, followed by administration of only reactive peptides (up to four peptides) as reported previously.[25-29] Selected peptides were mixed with incomplete Freund's adjuvant (Montanide ISA-51VG; Seppic, Paris, France), and four peptides of 1.5 ml emulsion each at doses of 3 mg/peptide were injected subcutaneously into the regional lymph node area. A total of 77 candidate peptides (32 peptides for HLA-A24-positive cancer patients, 37 for HLA-A2 and 8 for HLA-A3 supertypes) were used in the personalized peptide vaccination. All of these peptides can induce HLA-A24-, A2- and A3-supertype-restricted and tumor-specific CTL activity in PBMCs of cancer patients.[6,13-29,42-44]

Before the first vaccination and 7 days after every sixth vaccination, 30 ml of peripheral blood was obtained and PBMCs were isolated by means of Ficoll-Conray density gradient centrifugation. Peptide-specific CTL precursors in PBMCs were detected using the previously reported culture method.[25-29] Briefly, PBMCs (1 x 10^5 cells/well) were incubated with 10 μM of a peptide in 200 μl of culture medium in u-bottom 96-well microculture plates (Nunc, Roskilde, Denmark). Half of the medium was removed and replaced with a fresh 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 produce IFNγ in response to CIR-A2402 or T2 cells that were pre-loaded with either a corresponding peptide or HIV peptides (RYL RQQ LLG I for HLA-A24 and LLF GYP VYV for HLA-A2) as a negative control. For HLA-A3 supertype-positive cases, the cells were harvested and tested for their ability to produce IFNγ in response to CIR-A1101, -A31012 or -A3303 cells that were pre-loaded with either a corresponding peptide or an HIV peptide (RLR DLL LIV TR) as a negative control. The level of IFNγ was determined by enzyme-linked immunosorbent assay (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.

The levels of anti-peptide IgG were measured using the Luminex™ system, as previously reported.[25-29,45] In brief, plasma was incubated with 25 μl of peptide-coupled color-coded beads for 2 h at room temperature on a plate shaker. After incubation, the mixture was washed with a vacuum manifold apparatus and incubated with 100 μl of biotinylated goat anti-human IgG (chain-specific) for 1 h at room temperature. The plate was then washed, followed by the addition of 100 μl of streptavidin-PE to wells and 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 of Tween-PBS to each well. Fifty microliters of sample was used for detection with the Luminex™ system.

For evaluation of immune responses during the treatment, peptide-specific CTL precursors among PBMCs and serum levels of peptide-specific antibodies were measured every sixth vaccination. Positive immune responses were defined as either post (sixth vaccination) IgG levels/pre-IgG levels ≥ 2 or post (sixth vaccination) IFNγ levels/pre-IFNγ levels ≥ 2. In addition, in the analysis between long- and short-term survivors, positive immune responses were defined as either post (sixth vaccination) IgG levels/pre-IgG levels ≥ 10 or post (sixth vaccination) IFNγ levels/pre-IFNγ levels ≥ 10.

Adverse events and clinical responses. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. The clinical responses were evaluated on the basis of clinical observations and radiological findings. Patients were assigned a response category according to the Response Evaluation Criteria in Solid Tumors (RECIST).

Statistical methods. Overall survival and 1 and 3 year survival rates were determined by Kaplan-Meier actuarial analysis and the difference between survival curves was assessed by the log-rank test. Cox proportional hazards regression model was used for univariate and multivariate analyses to identify combinations of factors that had a significant impact on survival. All baseline parameters in the survival and proportional hazards regression analysis were analyzed as dichotomous variables using the overall mean values as cut-off levels. All statistical calculations were carried out using the StatView® program (SAS Institute Inc., Cary, NC). A two-sided significance level of 5% was considered statistically significant.

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Disclosure Statement

Although all authors completed the disclosure declaration, the following authors indicated a financial or other interest that is relevant to the subject matter under consideration in this article.

Employment or Leadership Position

Akira Yamada is a part-time executive of Green Peptide Co.; Consultant or Advisory Role: Kyogo Itoh, Green Peptide Co.; Stock Ownership: Kyogo Itoh, Akira Yamada, Green Peptide Co.; Honoraria: none; Research Funding: Kyogo Itoh, Akira Yamada, Green Peptide Co.; Expert Testimony: none; Other Remuneration: none.

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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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Grant sponsor: Green Peptide Co., Ltd.

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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$2$\times$ (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 ($\geq$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

			Anti-peptide IgG response (FIU)[a]				Anti-peptide cellular response (pg/ml)[b]			
Dose of peptide	Pts No.	Peptide	Pre	Post (fourth)	Post (after sixth)	Increased response (after sixth)	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]				Anti-peptide cellular response (pg/ml)[b]			
			Pre	Post (fourth)	Post (after sixth)	Increased response (after sixth)	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.

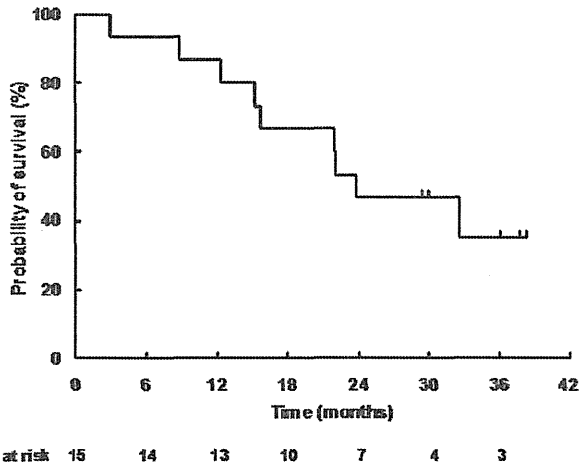

Fig. 1. Kaplan–Meier estimates of overall survival for 15 patients treated by personalized peptide vaccination with low-dose estramustine. Median overall survival is 23.8 months.

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

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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.

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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.

			SAE				
				Event no.			
Disease	n	Median age years	Observed case 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

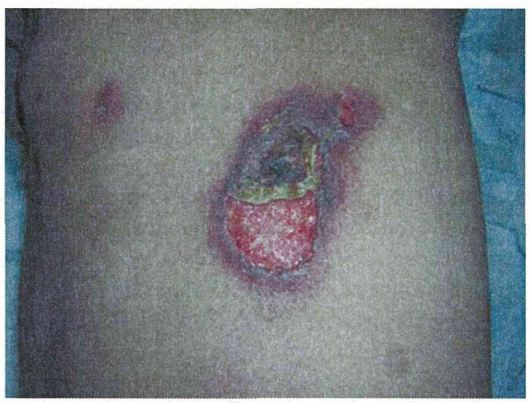

Figure 1. A skin ulcer at the injection site. Grade 3 ulcerations appeared at the previous injection sites of the thigh regions after the 19th vaccination in the abdominal region, in a patient with advanced pancreatic cancer (K-GEM-008).

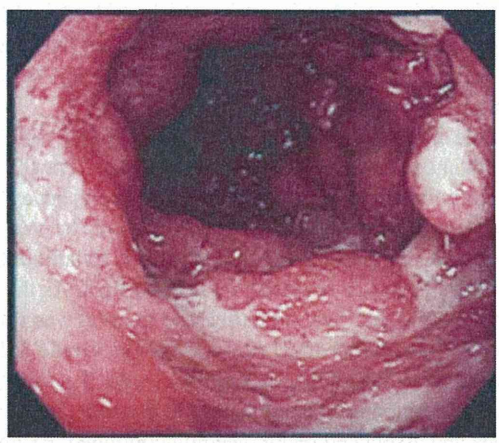

Figure 2. Colitis associated with ulcers. Examination with a sigmoid fiberscope revealed colitis associated with ulcers in a patient with advanced non-small cell lung cancer (EBL-002).

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

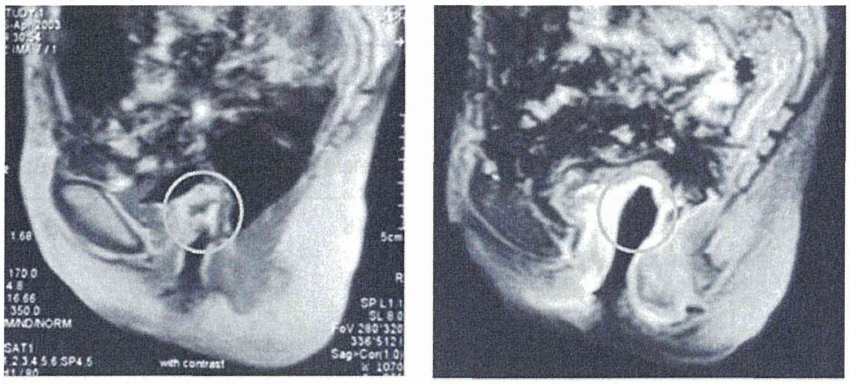

Figure 3. Bladder-vaginal fistula. Magnetic resonance imaging revealed the disappearance of the tumor mass after the 27th vaccination in a patient with advanced cervical cancer (GY-II-004).

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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