Cancer vaccines do not always elicit beneficial immune or clinical responses in treated patients. Therefore, identification of biomarkers for predicting clinical responses in vaccinated patients would be a significant issue in the clinical application of cancer vaccines (5,15-17). At present, however, there is little information available regarding predictive biomarkers in patients undergoing cancer vaccines. In this study, the multivariate analysis demonstrated that lower IL-6 and higher albumin values, which may reflect less inflammation and better nutritional status, prior to vaccination were significantly favorable factors for OS. IL-6 is a multifunctional cytokine that regulates various aspects of immune responses, acute phase reactions and hematopoiesis. In particular, IL-6 has been reported to be deeply involved in cancer development, such as tumor cell growth and cancer-associated inflammation (18).

There have been a number of studies describing the correlation between IL-6 levels and prognosis in various types of cancer (19-22). IL-6 has also been reported to be one of the critical cytokines for inducing suppressive immune cell subsets. For example, MDSCs and Th17, which are known to modulate antitumor immunity, were shown to be generated from their precursors in the presence of IL-6 and other cytokines (23-25). Although the role of IL-6 in the immune response to cancer vaccines remains to be clarified, it is possible that the blockage of IL-6 signaling would be beneficial for enhancing the therapeutic efficacy of cancer vaccines.

In conclusion, the present study demonstrated that PPV induced substantial immune responses to vaccine antigens without severe adverse events in advanced BTC patients. In addition, the multivariate analysis suggested that lower plasma IL-6 and better nutritional status prior to vaccination and pre-existing immune responses to greater numbers of antigens may contribute to better responses to PPV. Therefore, the evaluation of these factors prior to vaccination may be useful for selecting patients who would benefit from PPV and defining eligibility and/or exclusion criteria for molecular-based personalized immunotherapy in BTC patients. Nevertheless, since this was a small study with a limited number of patients, all of whom received PPV, the clinical efficacy of PPV, as well as the clinical utility of the identified factors in refractory BTC patients remain to be confirmed in future larger-scale prospective trials conducted in defined patient populations with or without receiving PPV.

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Immunological evaluation of personalized peptide vaccination in refractory small cell lung cancer

Yasuhiro Terazaki,[1] Koichi Yoshiyama,[1] Satoko Matsueda,[2] Noriko Watanabe,[2] Akihiko Kawahara,[3] Yoshiki Naito,[4] Shigetaka Suekane,[5] Nobukazu Komatsu,[2] Tetsuya Ioji,[2] Akira Yamada,[6] Takashi Mine,[1] Mizuhiko Terasaki,[7] Kyogo Itoh,[2] Shinzo Takamori[1] and Tetsuro Sasada[2,8]

[1]Departments of Surgery, and [2]Immunology and Immunotherapy, Kurume University School of Medicine, Kurume; [3]Department of Diagnostic Pathology, Kurume University Hospital, Kurume; [4]Departments of Pathology, and [5]Urology, Kurume University School of Medicine, Kurume; [6]Cancer Vaccine Division, Research Center of Innovative Cancer Therapy, Kurume University, Kurume; [7]Department of Neurosurgery, Kurume University School of Medicine, Kurume, Japan

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Since the prognosis of small cell lung cancer (SCLC) remains poor, development of new therapeutic approaches, including immunotherapies, would be desirable. In the current study, to evaluate immunological responses in refractory SCLC patients, we conducted a small scale phase II clinical trial of personalized peptide vaccination (PPV), in which vaccine antigens are selected based on pre-existing host immunity. Ten refractory SCLC patients, who had failed to respond to chemo- and/or chemoradiotherapies (median number of regimens, 2.5; median duration, 20.5 months), were enrolled. A maximum of four human leukocyte antigen (HLA)-matched peptides showing higher antigen-specific humoral responses were subcutaneously administered (weekly for six consecutive weeks and then bi-weekly thereafter). PPV was terminated before the 3rd administration in four patients because of rapid disease progression, whereas the remaining six patients completed at least one cycle (six times) of vaccinations. Peptide-specific immunological boosting was observed in all of the six patients at the end of the first cycle of vaccinations, with their survival time of 25, 24.5 (alive), 10 (alive), 9.5, 6.5, and 6 months. Number of previous chemotherapy regimens and frequency of $CD3^+CD26^+$ cells in peripheral blood were potentially prognostic in the vaccinated patients (hazard ratio [HR] = 2.540, 95% confidence interval [CI] = 1.188–5.431, P = 0.016; HR = 0.941, 95% CI = 0.878–1.008, P = 0.084; respectively). Based on the feasible immune responses in refractory SCLC patients who received at least one cycle (six times) of vaccinations, PPV could be recommended for a next stage of larger-scale, prospective clinical trials. (*Cancer Sci* 2012; 103: 638–644)

Although recent advances in chemotherapies contributed to improved clinical outcomes in refractory small cell lung cancer (SCLC) patients, their prognosis still remains very poor with a median survival time of 6–10 months.[1–3] Several clinical trials of immunotherapies have been attempted in refractory SCLC patients,[4,5] but none of them demonstrated a meaningful therapeutic benefit to patients. We have developed a novel regime of personalized peptide vaccination (PPV), in which vaccine antigens are selected and administered based on the pre-existing host immunity before vaccination.[6–13] For example, a recently conducted randomized clinical trial in advanced prostate cancer patients showed a promising clinical benefit of PPV.[7] In the current study, to address if refractory SCLC patients have the capability to respond to cancer vaccines, we conducted a small scale phase II study of PPV and evaluated immunological responses in the vaccinated patients.

Materials and Methods

Patients. Patients with histological diagnosis of SCLC were eligible for inclusion in the current study, if they had failed to respond to previous chemotherapies and/or chemoradiotherapies. They also had to possess positive humoral responses to at least two of the 31 different vaccine candidate peptides (Table S1), determined by both human leukocyte antigen (HLA) class I types and the titers of IgG against each peptide. The other inclusion criteria as well as exclusion criteria were not largely different from those of the previously reported clinical studies;[6–9] an age between 20 and 80 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; positive status for HLA-A2, -A3, -A11, -A24, -A26, -A31, or -A33; life expectancy of at least 12 weeks; adequate hematologic, renal, and hepatic function. Patients with lymphocyte counts of <1000 cells/μL were excluded from the study, since we previously reported that pre-vaccination lymphopenia is an un-favorable factor for overall survival (OS) in cancer patients receiving PPV.[11] Other exclusion criteria included pulmonary, cardiac, or other systemic diseases; an acute infection; a history of severe allergic reactions; pregnancy or nursing; or other inappropriate conditions for enrollment judged by clinicians. The protocol was approved by the Kurume University Ethical Committee and conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Tokyo 2004). It was registered in the UMIN Clinical Trials Registry (UMIN# 2984). After full explanation of the protocol, written informed consent was obtained from all patients before enrollment.

Clinical protocol. This was an open-label phase-II study, in which the primary and secondary endpoints were to identify biomarkers for OS and to evaluate safety in refractory SCLC patients who received PPV, respectively. Thirty-one peptides (PolyPeptide Laboratories, San Diego, CA, USA; American Peptide Company, Vista, CA, USA), whose safety and immunological effects had been confirmed in previously conducted clinical studies,[6–13] were used for vaccination (Table S1). The frequencies of expression of the parent proteins, from which the vaccine peptides were derived, in SCLC tissues were examined by immunohistochemistry (Fig. S1) and shown in Table S1. The right peptides for vaccination to individual patients were selected in consideration of the pre-existing host immunity before vaccination, assessed by the titers of IgG specific to each of the 31 different vaccine candidates, as previously described.[14] Although the prostate-related antigens, including prostate-specific antigen (PSA), prostatic acid

[8]To whom correspondence should be addressed.
E-mail: tsasada@med.kurume-u.ac.jp
Clinical trial registration information: UMIN Clinical Trials Registry (UMIN# 2984).

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phosphatase (PAP), and prostate-specific membrane antigen (PSMA), have been reported to be expressed not only by prostate cancer but also by other types of cancers,[15–18] the expression frequencies of these molecules in SCLC tissues were low (Table S2). Therefore, the peptides derived from them were selected only when pre-existing IgG responses to other remaining peptides were absent. A maximum of four peptides (3 mg/each peptide), which were selected based on the results of HLA typing and peptide-specific IgG titers, were subcutaneously administered with incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France) once a week for consecutive 6 weeks. After the first cycle of six vaccinations, up to four antigen peptides, which were re-selected according to the titers of peptide-specific IgG at every cycle of six vaccinations, were administered every 2 weeks up to four cycles (24 vaccinations). Combined chemotherapy and/or radiotherapy were allowed during the vaccination. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI-CTC Ver 3.0). The clinical responses were evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST 1.1) after the first cycle of vaccinations or at premature termination from the study. Pre-vaccination blood samples (PBMCs and plasma) were available from all of the enrolled patients (n = 10). Post-vaccination blood samples were available from six and four patients, who completed the first and second cycles of vaccinations, respectively.

Measurement of humoral and T cell responses. The humoral responses specific to each of the 31 peptide candidates (Table S1) were determined by peptide-specific IgG levels using the Luminex system (Luminex, Austin, TX, USA), as previously reported.[14] If the titers of peptide-specific IgG to at least one of the vaccine peptides in the post-vaccination plasma were more than twofold higher than those in the pre-vaccination plasma, the changes were considered to be significant.

T cell responses specific to the vaccine peptides were evaluated by interferon (IFN)-γ ELISPOT assay (MBL, Nagoya, Japan). Briefly, PBMCs (2.5×10^4 cells/well) were incubated in 384-well microculture plates (IWAKI, Tokyo, Japan) with 25 μL of medium (OpTmizer T Cell Expansion SFM; Invitrogen, Carlsbad, CA, USA) containing 10% FBS (MP Biologicals, Solon, OH), interleukin (IL)-2 (20 IU/mL; AbD serotec, Kidlington, UK), and each peptide (10 μM). Half of the medium was replaced with new medium containing the corresponding peptide (20 μM) at day 3. After incubation for the following 6 days, the cells were harvested and tested for their ability to produce IFN-γ in response to either the corresponding peptides or negative control peptides from human immunodeficiency virus (HIV). Antigen-specific IFN-γ secretion after 18-h incubation was determined by ELISPOT assay with an ELISPOT reader (ImmunoSpot S5 Versa Analyzer; Cellular Technology Ltd, Shaker Heights, OH, USA). Means of the triplicate samples were used for analyses. Antigen-specific T cell responses were evaluated by the differences between the spot numbers in response to the corresponding peptides and those to the control peptide; differences of at least 10 spot numbers per 10^5 PBMCs were considered as positive. If the spot numbers in response to at least one of the vaccine peptides in the post-vaccination PBMCs were more than twofold higher than those in the pre-vaccination PBMCs, the changes were considered as significant.

Measurement of C-reactive protein, serum amyloid A, and cytokines. C-reactive protein (CRP), serum amyloid A (SAA), and IL-6 in plasma were examined by ELISA using the kits from R&D systems (Minneapolis, MN, USA), Invitrogen, and eBioscience (San Diego, CA, USA), respectively. Multiplexed bead-based Luminex assays were used to measure Th1/Th2 cytokines, including IL-2, IL-4, IL-5, and IFN-γ (Invitrogen). Frozen plasma samples were thawed, diluted, and assayed in duplicate in accordance with the manufacturer's instructions. Means of the duplicate samples were used for analyses.

Flow cytometric analysis of immune cell subsets in PBMCs. A suppressive immune cell subset, myeloid-derived suppressor cells (MDSCs), in PBMCs was examined by flow cytometry. For analysis of MDSCs, PBMCs (0.5×10^6) were incubated for 30 min at 4°C with mAbs against lineage markers (CD3, CD14, CD19, CD56), CD33, and HLA-DR. In the cell subset negative for the lineage markers and HLA-DR, MDSCs were identified as positive for CD33. The frequency of MDSCs in the mononuclear cell gate defined by the forward scatter and side scatter was calculated. In addition, the expression of CD26 in PBMCs was analyzed, since the gene expression level of this molecule assessed by DNA microarray analysis was prognostic for OS in the prostate cancer patients receiving PPV (Sasada T , Komatsu N, Itoh K, unpublished observation). PBMCs were stained with anti-CD26 and anti-CD3 mAbs followed by calculation of the frequencies of $CD26^+$ subset in $CD3^+$ cells. The samples were run on a FACSCanto II (BD biosciences, San Diego, CA, USA), and data were analyzed using the Diva software (BD biosciences). All mAbs were purchased from Biolegend (San Diego, CA, USA).

Immunohistochemistry. Anti-tumor immune responses were examined by immunohistochemistry (IHC) in tumor tissues resected from SCLC patients treated with PPV (n = 1, Patient No. 5) or without PPV (n = 3). Paraffin-embedded tissue samples were cut into 4-μm sections, and labeled on the Bench-Mark XT (Ventata Automated Systems Inc., Tucson, AZ, USA) with anti-CD3 (clone LN10; Novocastra, Newcastle, UK), anti-CD4 (clone 4B12, Novocastra), and anti-CD8 (clone 4B11, Novocastra) mAb. The streptavidin-biotin complex method with 3,3′-diaminobenzidine tetrachloride (DAB) was used as a chromogen (Ventana iVIEW DAB Detection Kit). The expressions of vaccine antigens SART3 and p56lck in the tumor tissue from the patient treated with PPV (Patient No. 5) were also examined by IHC with anti-SART3 (rabbit polyclonal; Abcam, Cambridge, UK) and anti-p56lck (rabbit polyclonal, Abcam) Abs.

Statistical analysis. The Wilcoxon test was used to compare differences between pre- and post-vaccination measurements. All tests were two-sided, and differences at $P < 0.05$ were considered to be statistically significant. OS time was calculated from the first day of peptide vaccination until the date of death or the last date when the patient was known to be alive. Curves for OS were estimated by the Kaplan–Meier method. Potentially prognostic factors were evaluated by the Cox proportional hazards model. A value of $P < 0.1$ was used to identify potentially significant variables. All statistical analyses were conducted using the JMP version 9 or SAS version 9.1 software package (SAS Institute Inc., Cary, NC, USA).

Results

Patients' characteristics. Between March 2009 and October 2010, 10 patients with histology of SCLC were enrolled in this study. Table 1 shows the clinicopathological characteristics of the enrolled patients. All patients were male subjects with a median age of 63.5 years, ranging from 48 to 69. They had advanced stages of cancer (limited-stage disease [LD] at diagnosis, n = 5; extended-stage disease [ED] at diagnosis, n = 5), which had been refractory to previous treatments. Before enrollment, they failed to respond to one (n = 3), two (n = 2), three (n = 2), or more than 4 (n = 3) regimen(s) of chemotherapies and/or chemoradiotherapies. Median duration of these preceding regimens prior to the PPV was 20.5 months, ranging from 1 to 51. Performance status at the time of enrollment was grade 0 (n = 7) or grade 1 (n = 3). The numbers of peptides

Table 1. Characteristics of the enrolled patients with refractory SCLC (n = 10)

Patient No.	HLA Type	Gender	Age	Stage at diagnosis	PS	No. previous regimens	Previous treatment period (months)	Disease location (tumor size) before vaccination	No. vaccinations	Combined therapy	Treatment response†	OS (days)
1	A2/A26	M	58	ED	0	2	32	Mediastinal LN (28 mm), cervical LN‡, brain‡	24	CBDCA, PTX	PD	771
2	A24	M	68	LD	0	3	26	Pleural dissemination‡	2	(–)	PD	17
3	A24	M	62	LD	0	4	19	Cervical LN‡, liver (13 mm)	11	VNR	PD	178
4	A24/A26	M	52	ED	1	6	22	Liver (30 mm), bone (spine)‡, atelectasis‡	2	CBDCA, PTX	PD	16
5	A31/A33	M	67	LD	0	1	51	Lung (36 mm), brain‡	24	CDDP, VP16, WBRT	SD	746§
6	A2/A26	M	51	ED	0	2	5	Mediastinal LN‡, bone (spine)‡	10	AMR	Non-CR/ non-PD	285
7	A26/A31	M	65	LD	0	5	31	Lung (39 mm), adrenal (40 mm, 18 mm), brain (10 mm), mediastinal LN‡	2	CPT11, PTX	PD	33
8	A2/A24	M	69	ED	1	3	10	Pancreas (19 mm), mediastinal LN (15 mm)	14	(–)	PD	195
9	A2/A26	M	69	ED	1	1	3	Lung (50 mm), brain‡	1	(–)	PD	89
10	A2/A24	M	48	LD	0	1	1	Mediastinal LN (16 mm)	21¶	AMR, TPT, SRT	SD	306§

†Evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST 1.1). ‡Non-measurable lesion. §Patients alive (censored data). ¶Under treatment. AMR, amrubicin; CBDCA, carboplatin; CDDP, cisplatin; CPT11, irinotecan; CR, complete response; ED, extensive-stage disease; LD, limited-stage disease; LN, lymph node; M, male; OS, overall survival; PD, progressive disease; PS, performance status; PTX, paclitaxel; SCLC, small cell lung cancer; SD, stable disease; SRT, stereotactic radiotherapy; TPT, topotecan; VNR, vinorelbine; VP16, etoposide; WBRT, whole brain radiotherapy.

vaccinated to the patients at the first cycle of vaccinations were four peptides in eight patients and two in two patients. Of the 10 patients, six completed the first cycle of six vaccinations, whereas the remaining four patients failed before the 3rd vaccinations due to rapid disease progression. The median number of vaccinations was 10.5 with a range of 1–24. During the PPV, seven patients were treated in combination with chemotherapies and/or radiotherapy, and the remaining three patients did not tolerate them. None had a complete response (CR) or partial response (PR). The best response, seen in two patients, was stable disease (SD), whereas seven patients had progressive disease (PD). A patient without measurable lesions (Patient No. 6) had Non-CR/non-PD.

Toxicities. Toxicities are shown in Table 2. The most frequent adverse events were dermatological reactions at injection sites (n = 7), hematological toxicity (n = 10), and hypoalbuminemia (n = 8). Grade 3 serious adverse events (SAE) were as follows: dyspnea (n = 1), anemia (n = 1), leukocytopenia (n = 1), and lymphopenia (n = 1). The Grade 3 hematological SAE, including anemia, leukocytopenia, and lymphopenia, were transiently observed in the Patient No. 1 during PPV, just after he started receiving a concomitant chemotherapy with carboplatin and paclitaxel. But these SAE disappeared soon after stopping the concomitant chemotherapy, and did not recur even if he restarted the vaccinations after his recovery from the SAE. In addition, he showed no hematological SAE before this episode, while he received no concomitant chemotherapies. Based on these observations, the independent safety evaluation committee for this trial concluded that these SAE might not be directly associated with the vaccinations, but with the concomitant chemotherapy. The Grade 3 dyspnea was observed in Patient No. 2, who rapidly developed pleural effusion due to pleural dissemination and required hospitalization for oxygen supplementation. Since this symptom was highly likely to be caused by the rapidly progressing disease, the independent safety evaluation committee concluded that it might not be directly associated with the vaccinations.

Immune responses to the vaccine peptides. Both IgG and T cell responses specific to the vaccine peptides were analyzed in blood samples before and after vaccinations (Table 3). Plasma samples were obtained from 10, six and four patients before and at the end of the first (six vaccinations) and second (12 vaccinations) cycles of vaccinations, respectively. For monitoring of humoral responses, the titers of peptide-specific IgG reactive to each of 31 different peptides were measured by bead-based multiplex assay. The IgG responses specific to at least one of the vaccine peptides were augmented in five of six patients (83%) and in all of four patients (100%) examined at the end of the first and second cycles of vaccinations, respectively.

T cell responses to the vaccine peptides were also measured by IFN-γ ELISPOT assay (Table 3). PBMCs were available

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Table 2. Toxicities

	Grade 1	Grade 2	Grade 3	Grade 4	Total
Injection site reaction	3	4	0	0	7
Constitutional symptom					
Fever	0	1	0	0	1
Fatigue	2	0	0	0	2
Gastrointestinal					
Anorexia	2	0	0	0	2
Nausea	1	0	0	0	1
Pulmonary/Upper respiratory					
Dyspnea	0	0	1	0	1
Blood/Bone marrow					
Anemia	8	1	1	0	10
Leukocytopenia	3	0	1	0	4
Neutropenia	0	1	0	0	1
Lymphopenia	3	0	1	0	4
Thrombocytopenia	1	0	0	0	1
Laboratory					
AST elevation	0	1	0	0	1
ALT elevation	1	1	0	0	2
γ-GTP elevation	1	0	0	0	1
Creatinine elevation	1	1	0	0	2
Hypoalbuminemia	8	0	0	0	8
Hyperkalemia	1	0	0	0	1
Hyponatremia	1	0	0	0	1
Hyperglycemia	1	0	0	0	1
Hyperuricemia	1	0	0	0	1

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GTP, glutamyl transpeptidase.

from 10, six and three patients before and at the end of the first and second cycles of vaccinations, respectively. Antigen-specific T cell responses to at least one of the vaccine peptides were detectable in eight of 10 patients (80%) before vaccination, and augmented in five of six patients (83%) and in all of three patients (100%) tested at the end of the first and second cycles of vaccinations, respectively.

Collectively, at the end of the first cycle of six vaccinations, peptide-specific immunological boosting assessed by IgG and/or T cell responses was observed in all of the six patients who received at least six vaccinations, with their survival time of 25, 24.5 (alive), 10 (alive), 9.5, 6.5, and 6 months.

Cytokines and inflammation markers. We then measured cytokines (IL-2, IL-4, IL-5, IL-6, and IFN-γ) and inflammation markers (CRP and SSA) in the plasma before and at the end of the first cycle of vaccinations (Table 4). IL-6 was detectable in five of 10 patients (50%) before vaccination with median of 0.5 pg/mL, ranging from 0 to 7 pg/mL. IL-6 levels were increased, decreased, or unchanged in 2, 1, or 3 patients tested, respectively. There was no significant difference in the level of IL-6 between before and after vaccinations ($P = 0.500$; Wilcoxon test). Other cytokines, including IL-2, IL-4, IL-5, and IFN-γ, were rarely detectable in either pre- or post-vaccination plasma (data not shown).

An inflammation marker, CRP, was detectable in pre-vaccination plasma from the majority of patients (nine of 10 patients [90%]), with median value of 0.46 mg/dL (ranging from 0 to 1.04 mg/dL). Plasma CRP levels were increased or decreased in four or two patients, respectively. Another inflammation marker, SAA, was also detected in pre-vaccination plasma from all of the patients (100%) with median value of 5.475 mg/dL (ranging from 0.13 to 15.37 mg/dL). Plasma SAA levels were increased or decreased in three or three patients, respectively. There were no significant differences in the levels of CRP as well as SAA between before and after vaccinations ($P = 0.910$ and $P = 0.924$, respectively; Wilcoxon test).

Table 3. Immunological responses to the vaccine peptides

Patient No.	Peptide	IgG response†			T cell response‡		
		Before	1st	2nd	Before	1st	2nd
1	Lck-422	185	252	0	0	1000	2050
	HNRPL-140	428	723	1155	0	119	447
	SART3-109	224	657	2028	1309	294	186
	WHSC2-103	554	1332	16987	0	264	543
	MAP-432§	176	290	0	0	53	949
2	SART2-93	6609	NA	NA	0	NA	NA
	PSA-248	8975	NA	NA	0	NA	NA
	SART2-161	7979	NA	NA	0	NA	NA
	PSMA-624	7555	NA	NA	0	NA	NA
3	SART2-93	80	0	NA	146	0	NA
	MRP3-503	410	3040	NA	0	2389	NA
	SART2-161	166	0	NA	125	0	NA
	Lck-486	76	413	NA	0	364	NA
	PAP-213§	0	146	NA	NA	NA	NA
	PSMA-624§	38	42	NA	NA	NA	NA
4	PAP-213	552	NA	NA	0	NA	NA
	PSMA-624	266	NA	NA	333	NA	NA
	MAP-432	200	NA	NA	1333	NA	NA
	WHSC2-103	591	NA	NA	0	NA	NA
5	SART3-734	2142	11371	54795	1833	188	5390
	Lck-449	45	31	21708	600	944	9500
	SART3-109§	0	50	1854	NA	NA	0
	SART3-511§	0	28	1328	NA	NA	107
6	MAP-432	43	0	NA	0	227	NA
	HNRPL-501	104	446	NA	0	444	NA
	UBE2V-43	241	0	NA	157	71	NA
	SART3-109	2075	2621	NA	0	694	NA
7	SART3-109	174	NA	NA	117	NA	NA
	SART3-511	25	NA	NA	42	NA	NA
	Lck-90	85	NA	NA	0	NA	NA
	HNRPL-501	294	NA	NA	41	NA	NA
8	SART2-93	20	22	9222	0	56	NA¶
	PAP-213	208	187	12293	86	0	NA¶
	PSA-248	25	3856	18849	6	33	NA¶
	Lck-486	35	67	17704	15	16	NA¶
9	CypB-129	136	NA	NA	121	NA	NA
	Lck-422	34	NA	NA	13	NA	NA
10	Lck-246	74	63	3725	0	729	515
	WHSC2-141	77	58	455	0	75	0
	PAP-213	25	0	16345	0	89	166
	Lck-486	41	0	1378	0	102	0
	CypB-129§	70	86	81	0	0	19
	HNRPL-140§	43	48	24	0	34	64

†Values indicate the fluorescence intensity unit (FIU) of plasma IgG reactive with the corresponding peptides before and after the 1st and 2nd cycles of vaccinations. The augmented IgG responses are underlined. ‡Values indicate the number of spots per 10^5 peripheral blood mononuclear cells (PBMCs) reactive with the corresponding peptides in IFN-γ ELISPOT assay before and after the 1st and 2nd cycles of vaccinations. When the number of spots was <10 per 10^5 PBMCs, the data are shown as "0". The augmented T cell responses are underlined. §Peptides used for the 2nd cycle of vaccinations. ¶PBMCs unavailable. NA, not assessed.

Flow cytometric analysis of immune subsets in PBMCs. Immune cell subsets in both pre-vaccination and post-vaccination PBMCs were examined by flow cytometry (Table 4). The median frequency of MDSCs in pre- and post-vaccination PBMCs was 0.2% (range from 0 to 0.8%, $n = 10$) and 0.3% (range from 0 to 0.9%, $n = 6$), respectively. The median

Table 4. Laboratory data before and after vaccination†

Patient No.	IL-6 (pg/mL)		CRP (mg/dL)		SAA (mg/dL)		MDSCs (%)		$CD3^+CD26^+$ (%)	
	Before	After	Before	After	Before	After	Before	After	Before	After
1	0	0	0.39	0.56	8.58	7.78	0.3	0.6	48.2	58.4
2	7	NA	0.92	NA	12.65	NA	0.1	NA	29.8	NA
3	3	1	0.54	0.52	3.10	0.00	0.0	0.0	15.3	24.6
4	0	NA	0.47	NA	1.17	NA	0.1	NA	21.0	NA
5	1	2	0	0.56	0.28	3.99	0.2	0.1	32.9	34.8
6	3	9	0.39	0.61	5.47	11.95	0.2	0.5	49.7	57.3
7	1	NA	0.40	NA	5.48	NA	0.8	NA	19.0	NA
8	0	0	1.04	0.17	12.36	6.73	0.6	0.9	51.1	39.0
9	0	NA	0.94	NA	15.37	NA	0.4	NA	15.6	NA
10	0	0	0.45	0.53	0.13	0.55	0.1	0.1	39.4	28.3

†Values before and after the 1st cycle of vaccinations are shown. CRP, C-reactive protein; MDSCs, myeloid-derived suppressor cells; NA, not assessed; SAA, serum amyloid A.

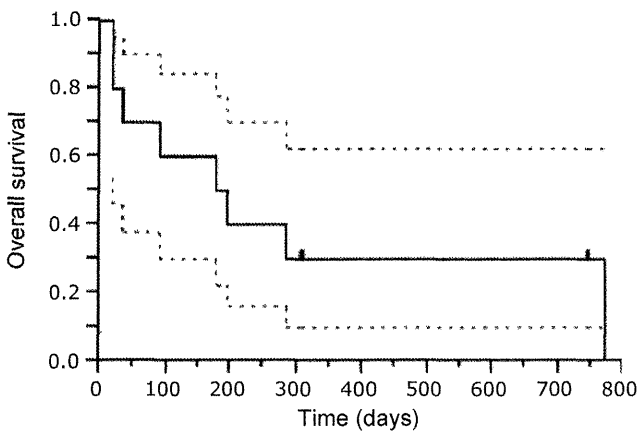

Fig. 1. Kaplan–Meier survival analysis in the enrolled patients. The median overall survival of patients who received personalized peptide vaccination (PPV) (n = 10; solid line) was 186.5 days and the 1 year survival rate was 30%. Dotted lines show 95% confidence intervals.

Table 5. Statistical analysis with clinical findings and laboratory data

Factor	Hazard ratio (95% CI)†	P-value†
Age	1.047 (0.943–1.163)	0.393
Limited-stage disease at diagnosis	1.250 (0.278–5.625)	0.771
Performance status (PS)	3.270 (0.651–16.427)	0.150
Number of previous treatment regimens	2.540 (1.188–5.431)	0.016
Previous treatment period (months)	0.989 (0.945–1.035)	0.637
Combined treatment (+)	0.336 (0.066–1.698)	0.187
IL-6 (pg/mL)	1.299 (0.900-1.877)	0.163
CRP (mg/dL)	7.459 (0.608–91.517)	0.116
SAA (mg/dL)	1.095 (0.940–1.275)	0.246
MDSCs (%)	2.872 (0.094–87.379)	0.545
$CD3^+CD26^+$ (%)	0.941 (0.878–1.008)	0.084

†Evaluated by the Cox proportional hazards model. CI, confidence interval; CRP, C-reactive protein; IL, interleukin; MDSCs, myeloid-derived suppressor cells; SAA, serum amyloid A.

frequency of $CD3^+CD26^+$ cells in pre- and post-vaccination PBMCs was 31.35% (range from 15.3 to 51.1%) and 36.9% (range from 24.6 to 58.4%), respectively. No significant differences were found in the frequencies of MDSCs and $CD3^+CD26^+$ between before and after the vaccinations (P = 0.140 and P = 0.825, respectively; Wilcoxon test).

Potentially prognostic factors in SCLC patients undergoing PPV. Median OS of the 10 patients was 186.5 days, with 1 year survival rate of 30% (Fig. 1). To identify potentially prognostic factors in refractory SCLC patients undergoing PPV, statistical analyses were carried out by the Cox proportional hazards model with clinical findings or laboratory data. As shown in Table 5, the number of previous chemotherapy regimens and frequency of $CD3^+CD26^+$ cells in PBMCs before vaccination were potentially prognostic in the patients receiving PPV (hazard ratio [HR] = 2.540, 95% confidence interval [CI] = 1.188–5.431, P = 0.016; HR = 0.941, 95% CI = 0.878–1.008, P = 0.084; respectively).

Accumulation of tumor-infiltrating lymphocytes in a patient undergoing tumor resection after PPV. A patient (Patient No. 5), who had good immune responses to vaccine antigens and showed stable disease (24.5 months alive), underwent resection of the primary tumor after 24 vaccinations. The parent proteins for the used peptides, SART3 and p56lck, were expressed in the tumor tissue resected after the vaccinations (Fig. 2). To know the immune responses to the tumor following the vaccinations, tumor-infiltrating lymphocytes were assessed by IHC using antibodies specific to immunological markers, including CD3, CD4, and CD8. In the tumor from this patient treated with PPV, $CD3^+$ cells infiltrated densely not only within the cancer stroma but also within the cancer cell nest (Fig. 3a). These tumor-infiltrating lymphocytes consisted of both $CD4^+$ and $CD8^+$ cells (Fig. 3b,c). In contrast, when the tumors from SCLC patients without PPV treatment (n = 3) were examined by IHC as a control, only a few cells positive for CD3, CD4, or CD8 accumulated within the tumors from all patients examined (representative data were shown in Fig. 3d–f). These results suggest the possibility that PPV induced anti-tumor immunity mediated by $CD4^+$ and $CD8^+$ T cells, leading to better clinical outcomes.

Discussion

Despite recent advances in chemotherapies for refractory SCLC patients, novel treatment modalities, including immunotherapies, still remain to be developed.[1–3] However, there have been a few reports available regarding immunotherapies against SCLC.[4,5] For example, a DC-based vaccine targeting p53 was reported to show a feasible result in a subset of SCLC patients, who had positive immune responses against p53. However, the induction rate of anti-p53 immunity was relatively low.[19,20] Vaccinations with cell surface glycolipid antigens to induce antigen-specific Ab responses were also attempted in several clinical studies.[21,22] However, only a

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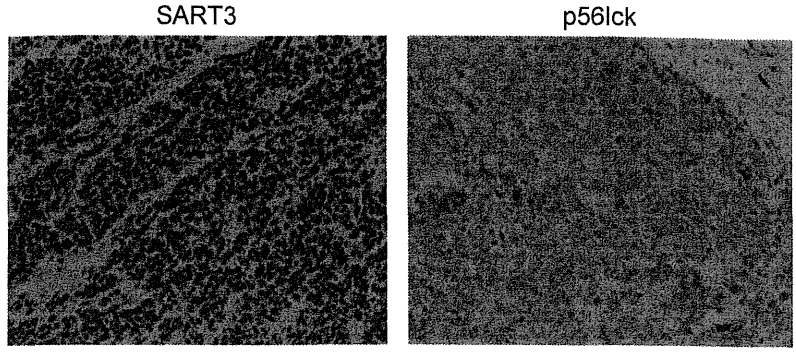

Fig. 2. Expression of the vaccine antigens in the tumor from a small cell lung cancer (SCLC) patient undergoing surgery after personalized peptide vaccination (PPV) treatment. The vaccine antigens SART3 and p56lck were detected by immunohistochemistry (IHC) with the antibodies specific to these molecules in the tumor tissue from a patient undergoing surgery after PPV treatment (Patient No. 5). Both sections, ×200.

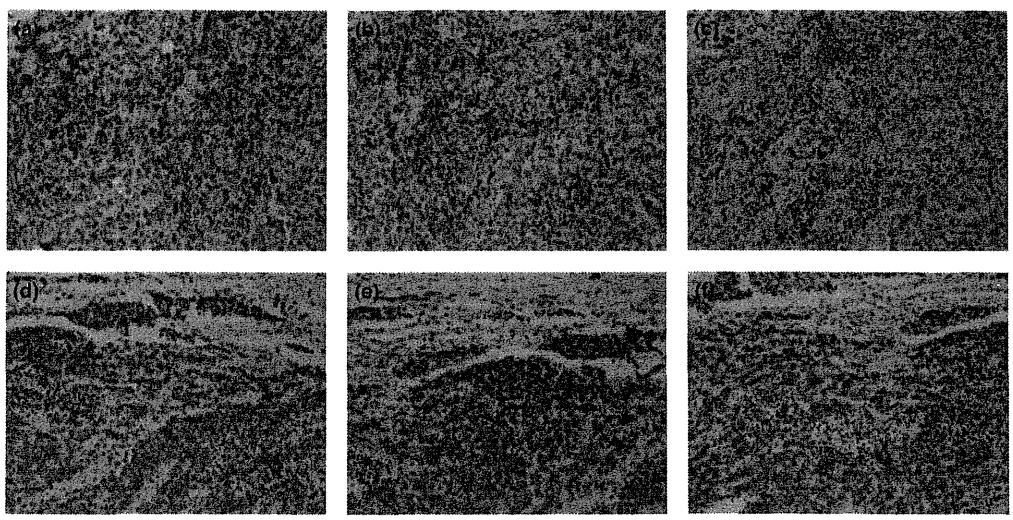

Fig. 3. Detection of tumor-infiltrating lymphocytes in tumors from small cell lung cancer (SCLC) patients treated with or without personalized peptide vaccination (PPV). Immune cells infiltrating within tumors were detected by immunohistochemistry (IHC) with the antibodies against CD3 (a and d), CD4 (b and e), and CD8 (c and f). All sections, ×100. (a–c) Tumor from a SCLC patient after PPV treatment (Patient No. 5). (d–f) Tumor from a SCLC patient without PPV treatment. Since the tumors from three SCLC patients without PPV treatment showed similar findings, representative data are shown.

limited number of patients developed a detectable Ab response, and there was no impact on clinical outcomes. In the current study, we addressed if refractory SCLC patients could have pre-existing IgG responses to 31 different vaccine candidates and well respond to these peptide vaccines. Notably, our results demonstrated that pre-vaccination plasma from all of the refractory SCLC patients had detectable levels of IgG specific to the cancer vaccine candidates, suggesting that they had the capability to show secondary immune responses to vaccine antigens. Furthermore, immunological boosting of T cell or IgG responses was observed in all of the patients, who completed at least one cycle of six vaccinations. Toxicity of PPV was mainly skin reactions at injection sites, and no SAE directly associated with the vaccinations were observed. These findings suggest the feasibility of PPV for refractory SCLC.

Interestingly, in a patient undergoing tumor resection after PPV, both $CD4^+$ and $CD8^+$ T cells infiltrated densely not only within the cancer stroma but also within the cancer cell nest. Since the vaccine antigens SART3 and p56lck were expressed in the tumor cells, it may be possible that T cells specific to these molecules infiltrated and accumulated within tumors. SART3 was strongly and homogeneously expressed in the tumor cells, whereas expression of p56lck was weak and heterogeneous. This heterogeneous expression of p56lck may be attributed to the immune escape mechanism of tumor cells following PPV, although the pre-vaccination tumor tissue of this patient was unavailable to demonstrate this possibility.

The prognosis of refractory SCLC patients remains very poor with a median survival time of around 6–10 months.[1–3] Therefore, it could be worthwhile to discuss the clinical efficacy of PPV, although it was not the main objective of this study. In 10 refractory SCLC patients receiving PPV, the median OS was 186.5 days, with 1 year survival rate of 30%. In particular, six patients who received at least one cycle of six vaccinations survived for 25, 24.5 (alive), 10 (alive), 9.5, 6.5, and 6 months (median OS, 528 days), although survival time of the remaining four patients without completing six vaccinations was only 0.5, 0.5, 1, and 3 months (median OS, 25 days). Statistically analyses with clinical findings and laboratory data were performed to identify potentially prognostic factors, although the result was preliminary due to the small number of patients and its clinical utility needs to be confirmed in future studies. In the analysis of clinical findings, greater numbers of previous chemotherapy regimens might be associated with worse prognosis, suggesting that PPV should be considered before repeated failures of multiple chemotherapeutic regimens. Similar to our finding, the ability to mount an immune response to therapeutic vaccines was reported to be directly correlated with fewer prior chemotherapy regimens.[23] In addition, the statistical analysis with pre-vaccination laboratory data demonstrated that the frequency of $CD3^+CD26^+$

cells in PBMCs was potentially prognostic in patients receiving PPV. The frequency of $CD3^+CD26^+$ cells has not been previously reported as a biomarker in SCLC patients. CD26 is a cell surface glycoprotein that functions as a proteolytic enzyme, dipeptidyl peptidase IV (DPP IV), and has been reported to play a critical role in signal transduction.[24] Since this molecule is highly expressed on activated T cells,[24] the increased frequency of $CD3^+CD26^+$ might contribute to better immune responses against the vaccine antigens. The role of $CD26^+$ activated T cells in cancer vaccines remains to be determined.

In summary, the current study demonstrated that immune responses to the vaccine antigens were substantially induced without SAE in refractory SCLC patients who received at least one cycle (six times) of vaccinations. Nevertheless, due to the small number of patients and the short term of observation in this early phase trial, clinical efficacy of PPV for refractory SCLC remains to be confirmed in a next step of larger-scale, prospective trials.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Immunohistochemical analysis of vaccine antigens in small cell lung cancer (SCLC) tissues.

Table S1. Peptide candidates for cancer vaccination.

Table S2. Frequency of expression of vaccine antigens in small cell lung cancer (SCLC) tissues.

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Personalized peptide vaccination in patients with refractory non-small cell lung cancer

KOICHI YOSHIYAMA[1], YASUHIRO TERAZAKI[1], SATOKO MATSUEDA[2], SHIGEKI SHICHIJO[2], MASANORI NOGUCHI[3], AKIRA YAMADA[3], TAKASHI MINE[1], TETSUYA IOJI[2], KYOGO ITOH[2], KAZUO SHIROUZU[1], TETSURO SASADA[2] and SHINZO TAKAMORI[1]

Departments of [1]Surgery and [2]Immunology and Immunotherapy, Kurume University School of Medicine; [3]Research Center of Innovative Cancer Therapy, Kurume University, Kurume, Fukuoka, Japan

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Abstract. Since the prognosis of non-small cell lung cancer (NSCLC) remains poor, the development of novel therapeutic approaches, including cancer vaccines, is highly desirable. In the current study, we conducted a phase II study of personalized peptide vaccination (PPV), in which a maximum of 4 peptides were selected based on pre-existing humoral immune responses and administered subcutaneously (weekly for 6 consecutive weeks and bi-weekly thereafter) in refractory NSCLC patients. Forty-one refractory NSCLC patients (4 stage IIIb, 22 stage IV and 15 recurrent), who had failed to respond to chemotherapy and/or targeted therapy (median number of regimens, 3; median duration, 10 months), were enrolled. Median overall survival (OS) was 304 days with a one-year survival rate of 42% in the enrolled patients. The main toxicity of PPV was skin reactions at the injection sites, but no serious adverse events were observed. In order to identify potential biomarkers for predicting OS, pre-vaccination and post-vaccination clinical findings and laboratory data were retrospectively assessed and evaluated by multivariate Cox regression analysis. Among the pre-vaccination factors examined, high C-reactive protein (CRP) level was a significant predictor of unfavorable OS [hazard ratio (HR)=10.115, 95% confidence interval (CI)=2.447-41.806, P=0.001]. Among the post-vaccination factors, high CRP level and low frequency of $CD3^+CD26^+$ cells were significant predictors of unfavorable OS (HR=23.127, 95% CI=2.919-183.233, P=0.003; HR=0.952, 95% CI=0.917-0.989, P=0.012). Taken together, our results suggest the feasibility of PPV for the treatment of refractory NSCLC. Evaluation of the identified factors before or at an early stage of vaccination could be potentially useful for selecting NSCLC patients who would likely have better prognosis following PPV.

Correspondence to: Dr Tetsuro Sasada, Department of Immunology and Immunotherapy, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan
E-mail: tsasada@med.kurume-u.ac.jp

Key words: non-small cell lung cancer, peptide vaccine, biomarker

Introduction

Non-small cell lung cancer (NSCLC) is one of the most common causes of cancer death worldwide. Although recent advances in chemotherapy and/or targeted therapy have helped to improve the clinical outcomes of patients with refractory NSCLC (1-5), their prognosis still remains very poor with a median survival time of 6-8 months. Therefore, development of novel therapeutic approaches, including cancer vaccines, would be highly desirable.

We developed a new approach of peptide-based vaccination, named personalized peptide vaccination (PPV), in which vaccine antigens are selected and administered based on pre-existing host immunity before vaccination (6-14). We have shown promising results of PPV in various types of advanced cancers (6-9). For example, a recently conducted randomized clinical trial of PPV for patients with advanced prostate cancer suggested a potentially favorable clinical outcome in the vaccinated group (9). However, to improve clinical efficacy further, prognostic biomarkers that would make it possible to select patients for whom cancer vaccines would be appropriate remain to be identified. In the present investigation, we conducted a small-scale phase II study to identify potential biomarkers that would be useful for prediction of overall survival (OS) before or at an early stage of vaccination in refractory NSCLC patients. Our results suggested the feasibility of PPV for refractory NSCLC. The identified factors would be informative for predicting the subpopulation of NSCLC patients, who would likely have better prognosis following PPV.

Patients and methods

Patients. Patients with a histological diagnosis of NSCLC were eligible for inclusion in the present study, if they had failed to respond to previous chemotherapy and/or targeted therapy. They also had to show positive humoral responses to at least two of the 31 different candidate vaccine peptides (Table I), determined by both HLA class I type and the titer of IgG against each peptide. The other inclusion criteria, as well as the exclusion criteria, were not largely different from those of other previously reported clinical studies (6-9): patient age

between 20 and 80 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 1 or 2; positive status for HLA-A2, -A3, -A11, -A24, -A26, -A31, or -A33; life expectancy of at least 12 weeks; negative status for hepatitis virus B and C; adequate hematologic, renal, and hepatic function. Patients with lymphocyte counts of <1000 cells/μl were excluded from the study, since we had previously reported that pre-vaccination lymphopenia is a predictor of unfavorable OS in cancer patients receiving PPV (12). Other exclusion criteria included pulmonary, cardiac, or other systemic diseases; acute infection; a history of severe allergic reactions; pregnancy or nursing; or other inappropriate conditions for enrollment as judged by clinicians. The protocol was approved by the Kurume University Ethics Committee, and was registered in the UMIN Clinical Trials Registry (UMIN no. 1839). After a full explanation of the protocol, written informed consent was obtained from all patients before enrollment.

Clinical protocol. This was an open-label phase II study in which the primary and secondary endpoints were to identify potential biomarkers for OS and to evaluate the safety of PPV in NSCLC patients, respectively. Thirty-one peptides, whose safety and immunological effects had been confirmed in previously conducted clinical studies (6-9, 13), were employed for vaccination [12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for HLA-A3 supertype (-A3, -A11, -A31, and -A33), 4 peptides for HLA-A26] (Table I). The peptides were prepared under the conditions of good manufacturing practice (GMP) by the PolyPeptide Laboratories (San Diego, CA) and American Peptide Company (Vista, CA). Appropriate peptides for vaccination in individual patients were selected in consideration of pre-existing host immunity before vaccination, assessed from the titers of IgG specific to each of the 31 different vaccine candidates, as described previously (14). Combined chemotherapy and/or targeted therapy were allowed during the vaccination period, unless patients were unable to tolerate combined chemotherapies or declined them (Table II). A maximum of 4 peptides (3 mg/each peptide), which were selected on the basis of HLA typing and peptide-specific IgG titers, were administrated subcutaneously with incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France) once a week for 6 consecutive weeks. After the first cycle of 6 vaccinations, up to 4 antigen peptides, which were re-selected according to the titers of peptide-specific IgG in every cycle of 6 vaccinations, were administered every 2 weeks. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI-CTC Ver3). Complete blood counts and serum biochemistry tests were performed after every 6 vaccinations. The clinical responses were evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) in the vaccinated patients, for whom computed tomography (CT) scan or magnetic resonance imaging (MRI) data were available before and after the first cycle of vaccinations.

Measurement of humoral and T cell responses. The humoral responses specific to each of the 31 candidate peptides (Table I), including those employed and not employed for vaccination, were determined by the peptide-specific IgG levels using the Luminex system (Luminex, Austin, TX), as reported previously (14). If the plasma titers of peptide-specific IgG in response to at least one of the vaccinated peptides after vaccination were >2-fold higher than those before vaccination, the changes were considered to be significant.

T cell responses specific to the vaccine peptides were evaluated by interferon (IFN)-γ Elispot using peripheral blood mononuclear cells (PBMCs), which were separated by density gradient centrifugation from peripheral blood (30 ml) with Ficoll-Paque Plus (GE Healthcare; Uppsala, Sweden) and stored frozen until analysis. After thawing, PBMCs ($2.5x10^4$ cells/well) were incubated in 384-well microculture plates (Iwaki, Tokyo, Japan) with 25 μl of medium (OpTmizer™ T Cell Expansion SFM; Invitrogen, Carlsbad, CA) containing 10% FBS (MP Biologicals, Solon, OH), IL-2 (20 IU/ml; AbD Serotec, Kidlington, UK), and each peptide (10 μM). Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) after culture for 3 days. After incubation for a further 6 days, the cells were harvested and tested for their ability to produce IFN-γ in response to either the corresponding peptides or a negative control peptide from human immunodeficiency virus (HIV) sequence (SLYNTYATL for HLA-A2; RYLRQQLLGI for HLA-A24; RLRDLLLIVTR for HLA-A3 supertype; EVIPMFSAL for HLA-A26). Antigen-specific IFN-γ secretion after 18 h of incubation was determined by Elispot, in accordance with the manufacturer's instructions (MBL, Nagoya, Japan). All assays were carried out in triplicate, and analyzed with the Zeiss Elispot reader (Carl Zeiss MicroImaging Japan, Tokyo, Japan). Antigen-specific T cell responses were evaluated by the difference between the numbers of spots produced in response to each corresponding peptide and that produced in response to the control peptide; a difference of at least 30 spots per 10^5 PBMCs was considered positive.

Measurement of C-reactive protein (CRP), serum amyloid A (SAA), and cytokines. CRP, SAA, and IL-6 in plasma were examined by ELISA using kits from R&D Systems (Minneapolis, MN), Invitrogen, and eBioscience (San Diego, CA), respectively. Multiplexed bead-based Luminex assays were used to measure Th1/Th2 cytokines, including IL-2, IL-4, IL-5, and IFN-γ (Invitrogen). Frozen plasma samples were thawed, diluted, and assayed in duplicate in accordance with the manufacturer's instructions.

Flow cytometric analysis of immune subsets among PBMCs. A suppressive immune subset, myeloid-derived suppressor cells (MDSCs), among PBMCs were examined by flow cytometry. For analysis of MDSCs, PBMCs ($0.5x10^6$) suspended in PBS containing 2% FBS were incubated with the following monoclonal antibodies (Abs) for 30 min at 4°C: anti-CD3-FITC, anti-CD56-FITC, anti-CD19-FITC, anti-CD33-APC, anti-HLA-DR-PE/Cy7, and anti-CD14-APC/Cy7. In the cell subset negative for lineage markers (CD3, CD19, CD56, CD14) and HLA-DR, MDSCs were identified as positive for CD33. The frequency of MDSCs in the lymphocyte gate defined by forward scatter and side scatter was calculated. In addition, the expression of CD26 in PBMCs was also analyzed, since the expression level of this gene assessed by cDNA microarray analysis has been shown to be predictive of OS in patients with prostate cancer receiving PPV (Sasada *et al*, unpublished data).

Table I. Peptide candidates for cancer vaccination.

Symbol for peptide	Protein	Position of peptide	Amino acid sequence	HLA type
CypB-129	Cyclophilin B	129-138	KLKHYGPGWV	A2, A3sup[a]
Lck-246	p56Lck	246-254	KLVERLGAA	A2
Lck-422	p56Lck	422-430	DVWSFGILL	A2, A3sup
MAP-432	ppMAPkkk	432-440	DLLSHAFFA	A2, A26
WHSC2-103	WHSC2	103-111	ASLDSDPWV	A2, A3sup, A26
HNRPL-501	HNRPL	501-510	NVLHFFNaPL	A2, A26
UBE-43	UBE2V	43-51	RLQEWCSVI	A2
UBE-85	UBE2V	85-93	LIADFLSGL	A2
WHSC2-141	WHSC2	141-149	ILGELREKV	A2
HNRPL-140	HNRPL	140-148	ALVEFEDVL	A2
SART3-302	SART3	302-310	LLQAEAPRL	A2
SART3-309	SART3	309-317	RLAEYQAYI	A2
SART2-93	SART2	93-101	DYSARWNEI	A24
SART3-109	SART3	109-118	VYDYNCHVDL	A24, A3sup, A26
Lck-208	p56Lck	208-216	HYTNaSDGL	A24
PAP-213	PAP	213-221	LYCESVHNF	A24
PSA-248	PSA	248-257	HYRKWIKDTI	A24
EGFR-800	EGF-R	800-809	DYVREHKDNI	A24
MRP3-503	MRP3	503-511	LYAWEPSFL	A24
MRP3-1293	MRP3	1293-1302	NYSVRYRPGL	A24
SART2-161	SART2	161-169	AYDFLYNYL	A24
Lck-486	p56Lck	486-494	TFDYLRSVL	A24
Lck-488	p56Lck	488-497	DYLRSVLEDF	A24
PSMA-624	PSMA	624-632	TYSVSFDSL	A24
EZH2-735	EZH2	735-743	KYVGIEREM	A24
PTHrP-102	PTHrP	102-111	RYLTQETNKV	A24
SART3-511	SART3	511-519	WLEYYNLER	A3sup
SART3-734	SART3	734-742	QIRPIFSNR	A3sup
Lck-90	p56Lck	90-99	ILEQSGEWWK	A3sup
Lck-449	p56Lck	449-458	VIQNLERGYR	A3sup
PAP-248	PAP	248-257	GIHKQKEKSR	A3sup

[a]A3sup, HLA-A3 supertype (A3, A11, A31, and A33).

PBMCs were stained with anti-CD26-PE and anti-CD3-FITC Abs. The frequency of the $CD26^+$ subset among $CD3^+$ cells was calculated. The samples were run on a FACSCanto II (BD Biosciences, San Diego, CA), and data were analyzed using the Diva software package (BD Biosciences). All Abs were purchased from Biolegend (San Diego, CA).

Statistical analysis. The two-sided Wilcoxon test was used to compare differences between pre- and post-vaccination measurements at a significance level of $P<0.05$. OS time was calculated from the first day of peptide vaccination until the date of death or the last date when the patient was known to be alive. The survival curve was estimated by the Kaplan-Meier method. Predictive factors for OS were evaluated by univariate and multivariate analyses with the Cox proportional hazards regression model. Statistically significant ($P<0.05$) variables in the univariate analysis were included in the multivariate analysis. Spearman rank correlation index was also utilized to choose the variables for multivariate analysis. All statistical analyses were conducted using the JMP version 8 or SAS version 9.1 software package (SAS Institute Inc., Cary, NC).

Results

Patient characteristics. Between December 2008 and October 2010, 41 patients with refractory NSCLC were enrolled in this study. Table II shows the clinicopathological characteristics of the enrolled patients. There were 19 male and 22 female subjects with a median age of 63 years, ranging from 37 to 76 years. Histologically, the tumors comprised 32 adenocarcinomas, 5 squamous cell carcinomas, 2 adenosquamous cell carcinomas, 1 large cell carcinoma, and 1 pleomorphic carcinoma. The

Table II. Characteristics of the enrolled patients with refractory NSCLC (n=41).

Patient no.	Histology	HLA type	Gender	Age	Stage	PS	Previous treatment		Combined therapy	No. of vaccination	Treatment response	OS (days)
							No. of regimens	Period (months)				
1	Ad	A24	F	67	IV	0	1	2	CBDCA + PTX	24	SD	683
2	Ad	A26	F	56	R	0	5	16	S-1	24	SD	691
3	Ad	A11/A31	M	70	IV	0	1	5	-	6	PD	58
4	Ad	A24	F	69	IV	1	4	4	-	15	PD	225
5	Adsq	A2/A24	M	68	IIIb	0	3	5	Erlotinib	7	PD	95
6	Adsq	A24/A33	F	52	R	0	2	18	Erlotinib	6	NA	467
7	Ad	A2/A33	M	63	IV	0	1	1	-	4	NA	41
8	Ad	A2/A24	F	53	R	1	5	24	GEM	9	PD	159
9	Pleo	A24	M	55	R	0	2	6	DOC	3	NA	41
10	Ad	A2/A26	M	50	R	0	1	11	CBDCA + PTX	6	NA	422
11	Ad	A2/A24	M	57	IIIb	0	1	6	-	18	SD	354
12	Ad	A24	M	72	IV	0	1	4	-	22	SD	596[b]
13	Sq	A11/A33	F	53	IV	0	2	8	Gefitinib	6	SD	573[b]
14	Ad	A26	M	75	R	0	2	10	-	17	SD	366
15	Ad	A2	F	59	IV	0	3	10	Gefitinib	8	PD	291
16	Ad	A2	F	54	IV	1	4	24	CDDP + PEM	2	NA	304
17	Ad	A24	F	72	IV	0	1	25	-	11	SD	266
18	Ad	A2/A33	F	69	R	0	6	23	-	5	NA	51
19	Ad	A2/A31	F	76	R	0	3	4	-	6	NA	503[b]
20	Ad	A2/A11	M	61	IV	0	1	4	DOC	6	NA	431
21	Ad	A2/A11	F	65	R	0	1	3	Gefitinib	20[a]	SD	412[b]
22	Ad	A2/A11	M	50	IV	0	1	2	-	14	NA	356
23	Ad	A24/A33	M	67	R	0	3	9	-	17	SD	398[b]
24	Ad	A2/A3	M	70	IV	0	2	12	-	6	NA	230
25	Ad	A24/A33	F	68	IV	1	4	9	-	7	PD	81
26	Ad	A26/A33	F	65	IV	0	6	30	-	5	NA	208
27	Ad	A2/A26	F	70	IV	0	3	21	Erlotinib	11	SD	258
28	Ad	A24/A26	M	53	R	0	4	13	-	11	NA	189
29	Ad	A24	M	54	IV	0	5	13	-	8	PD	77
30	Ad	A24	M	37	R	0	2	10	PEM	14	PD	239[b]
31	Sq	A2/A24	M	64	IIIb	0	3	6	VNR	14[a]	NA	232[b]
32	Ad	A2/A24	F	59	R	0	3	43	Gefitinib	16[a]	SD	251[b]
33	Ad	A24	F	73	IIIb	0	10	72	-	11	PD	246[b]

Table II. Continued.

Patient no.	Histology	HLA type	Gender	Age	Stage	PS	Previous treatment		Combined therapy	No. of vaccination	Treatment response	OS (days)
							No. of regimens	Period (months)				
34	Sq	A2/A24	F	62	IV	0	1	2	-	4	NA	50
35	Ad	A26/A33	F	54	IV	0	3	17	Gefitinib	14[a]	NA	239[b]
36	Sq	A24/A11	M	60	IV	0	3	12	-	15[a]	NA	237[b]
37	LCC	A24/A26	M	70	IV	0	6	19	-	14[a]	SD	190[b]
38	Sq	A2	M	66	R	1	3	6	-	10	PD	127
39	Ad	A2/A30	F	57	IV	0	4	32	PEM + Gefitinib	13[a]	PD	181[b]
40	Ad	A24/A26	F	44	R	0	3	23	Erlotinib	12	SD	176[b]
41	Ad	A2/A26	F	57	IV	0	2	11	-	12[a]	SD	176[b]

[a]Under treatment, [b]patients alive. NSCLC, non-small cell lung cancer; Ad, adenocarcinoma; Adsq, adenosquamous carcinoma; LCC, large cell carcinoma; Ple, Pleomorphic carcinoma; Sq, squamous cell carcinoma: M, male; F, female; R, recurrent; PS, performance status: CBDCA, carboplatin; PTX, paclitaxel; GEM, gemcitabine; DOC, docetaxel; CDDP, cisplatin; PEM, pemetrexed; VNR, vinorelbine; SD, stable disease; PD, progressive disease; NA, not assessed; OS, overall survival.

patients' cancers were at the refractory stage (stage IIIb, n=4; stage IV, n=22; recurrent, n=15) when they had failed to respond to one (n=11), two (n=7), three (n=11), or >4 (n=12) regimen(s) of chemotherapy, targeted therapy, and/or a combination of them. The median duration of these preceding regimens prior to PPV was 10 months, ranging from 1 to 72 months. Performance status at the time of enrollment was grade 0 (n=36) or grade 1 (n=5). The numbers of peptides used for vaccination of the patients during the first cycle were 4 peptides in 31 patients, 3 in 5 patients, and 2 in 5 patients. Among the 41 patients, 35 completed the first cycle of 6 vaccinations, whereas the remaining 6 patients failed to do so due to rapid disease progression. The median number of vaccinations was 11, with a range of 2 to 24. Among the 25 vaccinated patients for whom both pre- and post-vaccination radiological findings were available, none had a complete response (CR) or partial response (PR). The best response, seen in 14 patients, was stable disease (SD); the remaining 11 patients had progressive disease (PD).

Toxicities. Toxicities are shown in Table III. The most frequent adverse events were skin reactions at the injection sites (n=28) and hypoalbuminemia (n=21). One grade 4 serious adverse event (SAE), anemia, was noted. Grade 3 SAEs comprised injection site reaction (n=2), fever (n=1), hemoptysis (n=1), anemia (n=1), lymphopenia (n=1), and thrombocytopenia (n=1). According to evaluation by the independent safety evaluation committee for this trial, all of these SAEs, except for two cases of grade 3 injection site reaction, were concluded to be not directly associated with the vaccinations, but with cancer progression or other causes.

Immune responses to the vaccine peptides. Both humoral and T cell responses specific to the vaccine peptides were analyzed using blood samples obtained before and after the PPV. Plasma samples were obtained from 41, 35 and 18 patients before vaccination and at the end of the first (6 vaccinations) and second (12 vaccinations) cycles, respectively. Due to disease progression, 6 patients failed to complete the first cycle of 6 vaccinations. For monitoring of humoral immune responses, peptide-specific IgG reactive with each of the 31 different peptides, including those employed and not employed for vaccination, were measured by bead-based multiplex assay. The IgG responses specific to at least one of the vaccine peptides were augmented in 17 of 35 patients (49%) and in all of the 18 patients (100%) examined at the end of the first and second cycles of vaccination, respectively (data not shown).

T cell responses to the vaccine peptides were measured by IFN-γ Elispot assay. PBMCs from 36, 32 and 9 patients were available for this assay before and at the end of the first (6 vaccinations) and second (12 vaccinations) cycles, respectively. In the pre-vaccination samples, antigen-specific T cell responses were detectable in only 8 patients (22%). Among the 32 patients at the end of the first cycle of vaccinations, 11 (34%) showed T cell responses to the vaccine peptides. Among the 9 samples at the end of the second cycle of vaccinations, T cell responses were observed in 5 patients (56%) (data not shown).

Collectively, an increase of peptide-specific IgG titers was observed in about half and in all of the vaccinated patients at the end of the first and second cycles, respectively. In contrast,

Table III. Toxicities.

Toxicity type	Grade 1	Grade 2	Grade 3	Grade 4
Skin reactions at injection sites (n=28)	10	16	2	0
Constitutional symptom				
Fever (n=3)	1	1	1	0
Pulmonary/upper respiratory				
Dyspnea (n=3)	1	2	0	0
Hemoptysis (n=1)	0	0	1	0
Blood/bone marrow				
Anemia (n=11)	9	0	1	1
Leukocytopenia (n=7)	5	2	0	0
Neutropenia (n=4)	3	1	0	0
Lymphopenia (n=12)	10	1	1	0
Thrombocytopenia (n=2)	1	0	1	0
Laboratory				
Hyperbilirubinemia (n=3)	1	2	0	0
AST elevation (n=3)	2	1	0	0
ALT elevation (n=4)	3	1	0	0
Hypoalbuminemia (n=21)	17	4	0	0
Creatinine elevation (n=1)	1	0	0	0

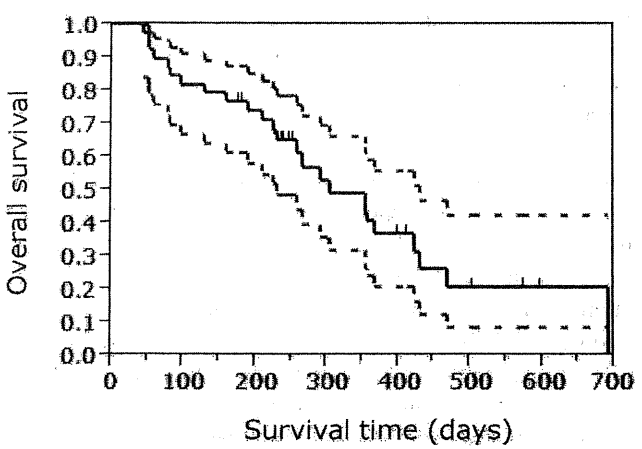

Figure 1. Kaplan-Meier survival analysis in the NSCLC patients receiving PPV. The median overall survival of patients who received PPV (n=41, solid line) was 304 days and the one-year survival rate was 42%. Dotted lines show 95% confidence intervals.

antigen-specific T cell responses were induced in only limited patients even after vaccination.

Cytokines and inflammation markers. We then measured cytokines (IL-2, IL-4, IL-5, IL-6, IFN-γ) and inflammation markers (CRP and SSA) in plasma before and at the end of the first cycle of vaccinations. IL-6 was detectable in 23 of 41 patients before vaccination, with a median level of 1 pg/ml, ranging from 0 to 103 pg/ml. Among the 35 plasma samples available at the end of the first cycle of vaccination, IL-6 levels were increased, decreased, and unchanged in 13, 7, and 15 patients, respectively. There was no significant difference in the level of IL-6 before and after vaccination (P=0.614, Wilcoxon test). However, the 22 patients who showed a decrease or no change in IL-6 levels after vaccination had a tendency to have a better prognosis than the remaining 13 patients who showed an increase in IL-6 (P=0.068, log-rank test). Other cytokines, including IL-2, IL-4, IL-5, and IFN-γ, were rarely detectable in either pre- or post-vaccination plasma (data not shown).

The inflammation marker, CRP, was detectable in pre-vaccination plasma from the majority of patients (40 of the 41 patients), with a median level of 0.39 mg/dl (ranging from 0 to 1.11 mg/dl). Among the 35 plasma samples tested at the end of the first cycle of vaccination, plasma CRP levels were increased and decreased in 30 and 5 patients, respectively. Another inflammation marker, SAA, was also detected in pre-vaccination plasma from the majority of patients (40 of the 41 patients), with a median level of 6.21 mg/dl (ranging from 0 to 14.12 mg/dl). Among the 35 plasma samples available at the end of the first cycle of vaccination, plasma SAA levels were increased and decreased in 25 and 10 patients, respectively. There were significant increases in the levels of CRP (P<0.001, Wilcoxon test) as well as SAA (P=0.005, Wilcoxon test) after vaccination, compared with those before vaccination. However, there were no significant associations between changes in CRP or SAA levels and clinical outcomes in the vaccinated patients (data not shown).

Flow cytometric analysis of immune subsets among PBMCs. Immune cell subsets among both pre-vaccination and post-vaccination PBMCs were examined by flow cytometry. The median frequency of MDSCs among pre- and post-vaccination PBMCs was 0.4% (range, 0.1-3.4%, n=33) and 0.3% (range, 0.1-2.0%, n=33), respectively. There was a significant decrease in the frequencies of MDSCs after vaccination (P=0.002, Wilcoxon

Table IV. Univariate and multivariate analysis with pre-vaccination clinical findings or laboratory data.

	Univariate analysis		Multivariate analysis	
Factor	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Age	1.006 (0.963-1.051)	0.786		
Gender	0.633 (0.281-1.428)	0.271		
Duration of previous treatment (months)	0.985 (0.934-1.039)	0.589		
Number of previous regimens	1.017 (0.807-1.282)	0.889		
Frequency of lymphocytes (%)	0.945 (0.898-0.993)	0.026		
Hemoglobin (g/dl)	0.826 (0.629-1.083)	0.167		
Albumin (g/dl)	0.220 (0.086-0.563)	0.002		
IL-6 (pg/ml)	1.021 (1.003-1.039)	0.020		
CRP (mg/dl)	9.375 (2.350-37.403)	0.002	10.115 (2.447-41.806)	0.001
Frequency of MDSCs (%)	1.089 (0.512-2.318)	0.825		
Frequency of $CD3^+CD26^+$ (%)	0.966 (0.914-1.021)	0.219		

CI, confidence interval; CRP, C-reactive protein; MDSCs, myeloid-derived suppressor cells.

Table V. Univariate and multivariate analysis with post-vaccination clinical findings or laboratory data.

	Univariate analysis		Multivariate analysis	
Factor	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Skin reactions at injection sites	0.861 (0.287-2.585)	0.789		
Increase in T cell responses	0.708 (0.227-2.203)	0.551		
Increase in humoral responses	1.042 (0.407-2.669)	0.932		
Frequency of lymphocytes (%)	0.953 (0.909-0.999)	0.048		
Hemoglobin (g/dl)	0.745 (0.546-1.017)	0.064		
Albumin (g/dl)	0.169 (0.064-0.445)	<0.001		
IL-6 (pg/ml)	1.055 (1.023-1.087)	<0.001		
CRP (mg/dl)	13.250 (2.095-83.794)	0.006	23.127 (2.919-183.233)	0.003
Frequency of MDSCs (%)	0.833 (0.183-3.785)	0.813		
Frequency of $CD3^+CD26^+$ (%)	0.956 (0.916-0.998)	0.042	0.952 (0.917-0.989)	0.012

CI, confidence interval; CRP, C-reactive protein; MDSCs, myeloid-derived suppressor cells.

test). The median frequency of $CD3^+CD26^+$ cells among pre- and post-vaccination PBMCs was 18.8% (range, 7.4-47.0%, n=35) and 18.3% (range, 3.3-61.8%, n=35), respectively. There was no significant difference in the frequencies of $CD3^+CD26^+$ cells (P=0.965, Wilcoxon test) before and after vaccination. There were no significant associations between changes in the frequencies of MDSCs or $CD3^+CD26^+$ cells and clinical outcomes in the vaccinated patients (data not shown).

Relationship between clinical findings or laboratory data and OS. The median OS for the 41 patients was 304 days, with a one-year survival rate of 42% (Fig. 1). The Cox proportional hazards model was used to identify factors that were significantly associated with OS from clinical findings or laboratory data before vaccination. Univariate analysis using pre-vaccination data showed that albumin, CRP, SAA, IL-6, and the frequency of lymphocytes in whole blood (P=0.002, P=0.002, P=0.004, P=0.020, and P=0.026, respectively) were significantly predictive of OS (Table IV). However, none of other factors examined, including age, gender, performance status, duration of chemotherapy or target therapy before vaccination, number of previous regimens, or other laboratory data (hemoglobin, creatinine, frequencies of regulatory T cells, MDSCs, or $CD3^+CD26^+$ cells), were significantly correlated with OS (data not shown). In addition, multivariate Cox regression analysis was performed to evaluate the influence of each of the factors that had been shown to be significantly associated with OS in the univariate analysis (P<0.05), after adjusting for possible confounding factors. Albumin, CRP, IL-6, and the frequency of lymphocytes in whole blood were included in the multivariate Cox regression analysis. SAA was excluded in this analysis, since the level of SAA was highly correlated with that of CRP (Spearman rank

correlation coefficient, 0.819; P<0.001). As shown in Table IV, higher CRP level in pre-vaccination plasma was significantly predictive of unfavorable OS [hazard ratio (HR)=10.115, 95% confidence interval (CI)=2.447-41.806, P=0.001]. However, the other factors showed no significant association.

Similarly, the Cox proportional hazards model was used to identify factors associated with OS from clinical findings or laboratory data at the end of the first cycle of vaccination. Univariate analysis showed that albumin, IL-6, SAA, CRP, frequency of $CD3^+CD26^+$ cells, and frequency of lymphocytes in whole blood were predictive of OS at the end of the first cycle of vaccination (P<0.001, P<0.001, P=0.004, P=0.006, P=0.042, and P=0.048, respectively) (Table V). None of the other factors, including other laboratory data, increase in IgG or T cell responses to the vaccine peptides, and skin reactions at the injection sites, were significantly correlated with OS. Albumin, IL-6, CRP, frequency of $CD3^+CD26^+$ cells, and frequency of lymphocytes were included in the multivariate Cox regression analysis. SAA was excluded in this analysis, since the level of SAA was highly correlated with that of CRP (Spearman rank correlation coefficient, 0.698; P<0.001). Multivariate Cox regression analysis demonstrated that higher CRP level and lower frequency of $CD3^+CD26^+$ cells in post-vaccination samples were predictive of unfavorable OS (HR=23.127, 95% CI=2.919-183.233, P=0.003; HR=0.952, 95% CI=0.917-0.989, P=0.012) (Table V).

Discussion

Since only a subset of patients obtain clinical benefits from peptide-based cancer vaccines, it would be critical to identify biomarkers for selection of suitable patients (15-17). With regard to post-vaccination biomarkers, we have shown that an increase in peptide-specific IgG responses after PPV is well associated with improved OS in patients with certain types of cancers (12,18). In addition, several factors, including cytotoxic T lymphocytes (CTL) responses, Th1 responses, delayed type hypersensitivity (DTH), and autoimmunity, have also been reported to be associated with clinical responses in some clinical trials (16,17,19,20), although these results have not always been reproducible. Notably, there are currently no validated pre-vaccination biomarkers, predictive of clinical responses, in widespread use. Therefore, in the present study, we searched for clinically useful predictive markers for PPV in patients with NSCLC. Multivariate analysis of pre-vaccination factors showed that higher level of plasma CRP was predictive of unfavorable OS. Among post-vaccination factors, higher level of plasma CRP and lower frequency of $CD3^+CD26^+$ cells were predictive of unfavorable OS. Although more data are still needed to validate our findings, evaluation of the factors identified here could be useful for selecting patients with NSCLC who would potentially benefit from cancer vaccines.

Elevated CRP level was shown to be also a predictor of unfavorable OS in NSCLC patients receiving chemotherapy or targeted therapy (21,22), suggesting that it might not necessarily be unique to vaccinated patients. In contrast, the frequency of $CD3^+CD26^+$ cells among PBMCs has not been reported previously as a biomarker in NSCLC patients. CD26 is a cell surface glycoprotein that functions as a proteolytic enzyme, dipeptidyl peptidase IV, and plays a critical role in signal transduction (23). Since it is highly expressed on activated T cells (23), increased frequency of $CD3^+CD26^+$ might reflect the immune activation induced by vaccination. The role of $CD26^+$ activated T cells induced by PPV in NSCLC thus remains to be determined.

MDSCs are a heterogeneous population of immature myeloid cells that inhibit the functions of other immune cells and promote tumor progression (24,25). MDSCs can facilitate tumor growth by inducing angiogenesis at tumor sites or by suppressing anti-tumor immune cells, such as antigen-specific T cells (24,25). Notably, the frequencies of MDSCs were significantly decreased after PPV. In addition, the patients who showed a decrease or no change in IL-6 after vaccination had a tendency to have better outcome. IL-6 is a multifunctional cytokine that regulates various aspects of cancer development, such as tumor cell growth and suppression of anti-tumor immune cells, including CTL and NK cells (26). The roles of these immune suppressive cells and/or cytokine, MDSCs and IL-6, in immune responses to cancer vaccines remain to be examined.

The prognosis of refractory NSCLC patients remains very poor, with a median survival time of 6-8 months (1-5). In contrast, the median OS of the 41 NSCLC patients who received PPV was 304 days (>10 months), with a one-year survival rate of 42%, in the current study. The main toxicity of PPV was skin reactions at the injection sites, but no SAEs were observed. Our previous trials of PPV for various types of cancers have also confirmed its safety (13). Considering the disease conditions of the patients enrolled in the current study, all of whom had already been resistant to or ineligible for conventional chemotherapeutic and targeted agents before enrollment, our findings suggest the feasibility of PPV for refractory NSCLC, even though OS was not the main objective of the current study. Nevertheless, since this is a retrospective study with a limited number of patients, clinical utility of PPV should be further verified in larger-scale, prospective trials conducted in defined patient populations with or without receiving PPV.

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Personalized peptide vaccination

A novel immunotherapeutic approach for advanced cancer

Tetsuro Sasada,[1,*] Masanori Noguchi,[2] Akira Yamada[2] and Kyogo Itoh[1]

[1]Department of Immunology and Immunotherapy; Kurume University School of Medicine; Kurume, Japan; [2]Research Center for Innovative Cancer Therapy; Kurume University; Kurume, Japan

Since both tumor cells and immune cell repertoires are diverse and heterogeneous, immune responses against tumor-associated antigens might be substantially different among individual patients. Personalized selection of right peptides for individuals could thus be an appropriate strategy for cancer vaccines. We have developed a novel immunotherapeutic approach, personalized peptide vaccination (PPV), in which HLA-matched peptides are selected and administered, based on the pre-existing host immunity before vaccination. Recent clinical trials of PPV have demonstrated a feasibility of this new therapeutic approach in various types of advanced cancers. For example, a randomized phase II trial for patients with castration resistant prostate cancer showed a possible clinical benefit in the PPV group. In the patients undergoing PPV, lymphocyte counts, increased IgG responses to the vaccine peptides, and inflammatory factors in pre-vaccination peripheral blood might be potential biomarkers for prognosis. Further randomized phase III trials would be recommended to prove clinical benefits of PPV.

Keywords: peptide vaccine, personalized vaccine, cytotoxic T lymphocytes, advanced cancer, biomarker, inflammation

Abbreviations: PPV, personalized peptide vaccination; CTL, cytotoxic T lymphocytes; CRPC, castration-resistant prostate cancer; FDA, food and drug administration; MST, median survival time; HR, hazard ratio; CI, confidence interval

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*Correspondence to: Tetsuro Sasada; Email: tsasada@med.kurume-u.ac.jp

Introduction

The field of cancer immunotherapy has drastically moved forward during these two decades since Boon and his colleagues reported for the first time a tumor-associated antigen, MAGE-A1, recognized by cytotoxic T lymphocyte (CTL) in 1991.[1] In particular, there have recently been noteworthy advances in the clinical application of cancer immunotherapy.[2,3] In 2010, sipuleucel-T (Provenge; Dendreon Corporation), an autologous cellular immunotherapy product designed to stimulate T cell immune responses against human prostatic acid phosphatase (PAP), was first approved for patients with castration-resistant prostate cancer (CRPC) by the US. Food and Drug Administration (FDA).[4] In addition, another immunotherapeutic agent, ipilimumab, an anti-cytotoxic T lymphocyte antigen (CTLA)-4 monoclonal antibody, was also approved for melanoma patients by the FDA in 2011.[5] Despite these significant advances, however, most of other randomized clinical trials in cancer immunotherapy have so far failed to show beneficial therapeutic effects compared with existing treatments.[6,7] The failure of recent clinical trials has raised several issues to be addressed for development of cancer vaccines. Here, we have proposed a novel immunotherapeutic approach, "personalized peptide vaccination (PPV)" for advanced cancer patients.

Rationale for Personalized Selection of Vaccine Antigens in Individual Cancer Patients

A large number of tumor-associated antigens have been identified by several different approaches, including cDNA expression cloning, serologic analysis of recombinant cDNA expression libraries (SEREX), and reverse immunological approach.[8] Although the number of cancer vaccine candidates is becoming almost limitless, antigens currently employed

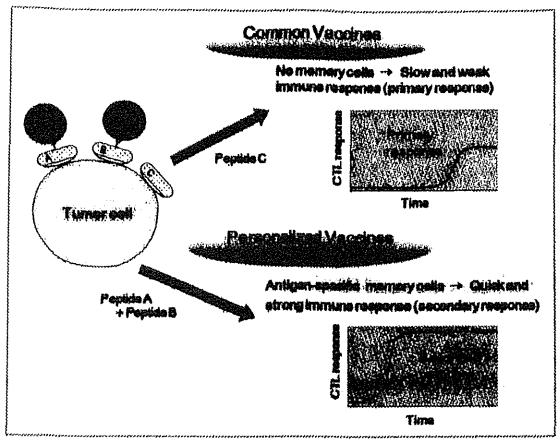


Figure 1. Personalized vaccines are more powerful than common vaccines. Personalized vaccines [illegible]

for vaccination against individual cancer patients might not always be appropriate. In general, anti-tumor immunity is known to be dependent on both immunological characters of tumor cells and immune cell repertoires. Since immune cell repertoires are quite diverse and heterogeneous, anti-tumor immunity might be substantially different among individuals. Therefore, it is likely that vaccine antigens that are selected and administered without considering the immune cell repertoires of the hosts could not efficiently induce beneficial anti-tumor immune responses. To increase the clinical benefits from cancer vaccines, particular attentions should be paid to immunological status of each patient by characterizing the pre-existing immune responses to vaccine antigens before vaccination.

Nevertheless, in most of current clinical trials of therapeutic cancer vaccines, common antigens are employed for vaccination independently of immunological status of patients. Patients, who have immunological memory to vaccine antigens, are expected to show quick and strong immune responses to them. In contrast, patients with no immunological memory against vaccine antigens would take more time for development of effective anti-tumor immune responses, because several rounds of repeated vaccinations might be required to prime antigen-specific naive T cells to functional effector cells (Fig. 1). In such situations, vaccinations could not easily provide clinical benefits, especially in advanced cancer patients, who show a relatively quick disease progression. Moreover, immune responses induced by inadequate vaccines that are non-specific to tumor cells may not only be ineffective for tumor control, but also erode pre-existing immunity.[9] Based on the current paradigm that the size and composition of the adaptive immune system are limited and that individual immune cells are constantly competing each other in the limited space, inadequate vaccination may have negative consequences for the hosts by suppressing pre-existing beneficial memory cells specific to tumors and/or infections, which might result in acceleration of cancer progression or early death in vaccinated patients.[10] Considering these issues, it would be quite reasonable that vaccine antigens should be selected based on the pre-existing immunological status in each patient.

In addition, it should be noted that cancer cells possess or develop a variety of mechanisms to maintain their malignant behavior. For example, it has been well recognized that cancer cells escape from host immunological surveillance.[11] Through the interaction between host immune system and tumor cells at the equilibrium phase, immunological pressure often produces tumor cell variants that decrease or lose tumor-associated antigens. Therefore, to better control cancer cells, it would be recommended to target multiple tumor-associated antigens to reduce the risk of outgrowth of antigen-loss variants.

PPV as a Novel Immunotherapeutic Approach

In view of complexity and diversity of immunological characters of tumors and immune cell repertoires, we have developed a new concept of PPV.[12] In this "personalized" cancer vaccine formulation, appropriate peptide antigens for vaccination are screened and selected from a list of vaccine candidates in each patient, based on pre-existing host immunity. Currently, we employ 31 HLA class I-restricted peptide candidates, which were identified from a variety of tumor-associated antigens mainly through cDNA expression cloning method with tumor-infiltrating lymphocyte clones/lines; 12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for HLA-A3 supertype (A3, A11, A31 or A33), and 4 peptides for HLA-A26. The safety and potential immunological effects of these vaccine candidates have been shown in previously conducted clinical studies.[12-14] A maximum of 4 peptides, which are selected based on the results of HLA typing and the pre-existing immune responses specific to each of the 31 different vaccine candidates, are subcutaneously administered in complex with incomplete Freund's adjuvant weekly or bi-weekly.

Currently, we evaluate the pre-existing immune responses to vaccine candidates by B cell responses, but not by T cell responses, since the performance characteristics, such as sensitivity and reproducibility, of current T cell assays are unsatisfactory.[3,15] In contrast to these drawbacks inherent to T cell assays, B cell assays have more potential for screening and/or monitoring antigen-specific immune responses even to MHC class I-restricted peptides.[3] Indeed, we have recently published several papers describing the clear correlations between clinical benefits and antigen-specific B cell responses measured by IgG antibody production in patient plasma after vaccination.[16] Notably, the multiplex bead-based LUMINEX technology that we have developed for monitoring B cell

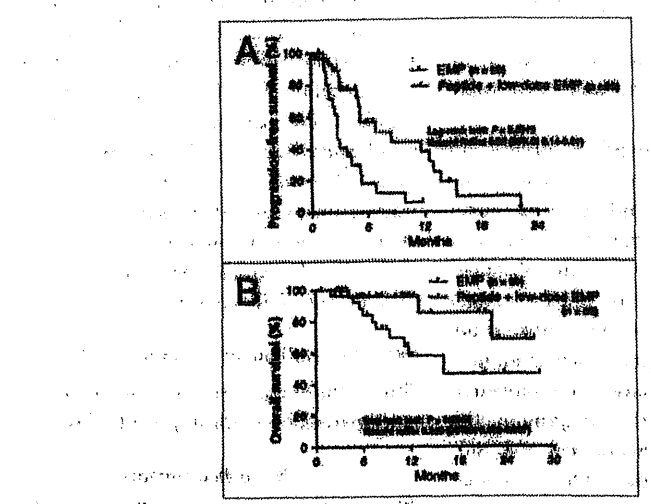
Table 1. Clinical responses of advanced cancer patients treated with PPV

			PR	SD	PD		
Prostatic	155	29	36	90	18.7	87.0
Colorectal	74	68	1	23	44	1.5	35.3
Gastric	42	35	0	8	27	0	22.9
Cervical	28	23	3	7	13	13.0	43.5
Renal cell	13	12	0	9	3	0	75.0
Breast	11	10	0	1	9	0	10.0
Others	31	23	0	8	15	0	34.8

Best clinical responses were evaluated by RECIST criteria (or PSA values in prostatic cancer). PR, partial response; SD, stable disease; PD, progressive disease.

responses allows simple, quick and highly reproducible high-throughput screening of IgG responses specific to large numbers of peptide antigens with a tiny amount of plasma.[17]

In the clinical trials of PPV conducted during the past several years, we have shown promising results in various types of cancers.[12,13,16,18,19] Table 1 shows the clinical responses in 500 advanced cancer patients who received PPV from October 2000 to October 2008.[16] The best clinical response assessed in 436 evaluable patients were partial response (PR) in 43 patients (10%), stable disease (SD) in 144 patients (33%) and progressive disease (PD) in 249 patients (57%), with a median overall survival of 9.9 mo. Of note, as shown in Figure 2, a recently conducted phase II randomized clinical trial of PPV for 57 CRPC patients demonstrated that patients receiving PPV in combination with low-dose estramustine phosphate (EMP) showed a significantly longer progression-free [median survival time (MST), 8.5 vs. 2.8 mo; hazard ratio (HR), 0.28 (95% confidence interval (CI), 0.14–0.61); p = 0.0012], and overall survival [MST, undefined vs. 16.1 mo; HR, 0.30 (95% CI, 0.10–0.91); p = 0.0328] than those receiving standard-dose EMP alone.[18] In addition, PPV was also conducted in an early phase clinical trial of patients with recurrent or progressive glioblastoma multiforme, one of the most aggressive brain tumors, with median overall survival of 10.6 mo.[19] Based on these promising results, randomized phase III trials are currently underway in CRPC and glioblastoma. To prove clinical benefits of PPV for accelerating cancer vaccine development, further randomized phase III trials would also be recommended in other different types of cancers.



Lymphocyte Counts, Increased Humoral Responses to the Vaccine Antigens, and Inflammatory Factors as a Biomarker for PPV

Only a subset of patients show clinical benefits from cancer immunotherapy, including peptide-based cancer vaccines. In addition, even worse, some large clinical trials in the past several years