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

	Univariate analysis		Multivariate analysis	
Factor	Hazard ratio (95% CI)	P-value[a]	Hazard ratio (95% CI)	P-value[a]
Age	0.986 (0.944-1.030)	0.523		
Gender	1.673 (0.586-4.776)	0.336		
Duration of previous chemotherapy (months)	1.056 (0.965-1.154)	0.235		
Lymphocyte count ($x10^3/mm^3$)	0.639 (0.202-2.023)	0.446		
Hemoglobin (g/dl)	0.618 (0.392-0.976)	0.039		
Albumin (g/dl)	0.158 (0.041-0.616)	0.008	0.158 (0.029-0.860)	0.033
IL-6 (pg/ml)	1.159 (1.055-1.274)	0.002	1.123 (1.008-1.252)	0.035
CRP (μg/ml)	1.533 (1.143-2.056)	0.004		
SAA (μg/ml)	1.014 (1.001-1.027)	0.031		
MDSC (%)	1.140 (0.823-1.580)	0.432		
Treg (%)	0.823 (0.561-1.206)	0.317		
No. of selected peptides	0.395 (0.163-0.953)	0.039	0.258 (0.098-0.682)	0.006

[a]P-values determined by the Cox proportional hazard regression model. CI, confidence interval; IL-6, interleukin-6; CRP, C-reactive protein; SAA, serum amyloid A; MDSC, myeloid-derived suppressor cells; Treg, $CD4^+CD25^+Foxp3^+$ regulatory T cells.

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

	Univariate analysis		Multivariate analysis	
Factor	Hazard ratio (95% CI)	P-value[a]	Hazard ratio (95% CI)	P-value[a]
Elevation of CTL responses	0.530 (0.166-1.691)	0.284		
Elevation of humoral responses	0.364 (0.114-1.165)	0.089		
Hemoglobin (g/dl)	0.668 (0.463-0.965)	0.031		
Albumin (g/dl)	0.173 (0.055-0.544)	0.003		
IL-6 (pg/ml)	1.112 (1.033-1.198)	0.005	1.152 (1.052-1.261)	0.002
CRP (μg/ml)	1.217 (1.023-1.448)	0.027		
SAA (μg/ml)	1.008 (0.995-1.021)	0.234		
No. of vaccinated peptides	0.271 (0.082-0.899)	0.033	0.120 (0.027-0.540)	0.006

[a]P-values determined by the Cox proportional hazard regression model. CI, confidence interval; IL-6, interleukin-6; CRP, C-reactive protein; SAA, serum amyloid A.

peptides were significantly favorable factors for OS (HR=1.152, 95% CI 1.052-1.261, P=0.002; HR=0.120, 95% CI 0.027-0.540, P=0.006; respectively) (Table V). However, the other post-vaccination factors were not significantly associated with OS.

Discussion

For patients with advanced or recurrent BTC that are ineligible for surgery, various regimens of chemotherapeutic agents have been investigated (1-4). For example, a combination of chemotherapeutic agents, such as gemcitabine and cisplatin, has recently demonstrated a promising result (3). However, further treatment modalities for refractory patients who are unresponsive to or relapse following such regimens remain to be established. This is the first clinical report of refractory BTC patients who received PPV. Immune responses to the vaccine antigens, which have been reported to be significantly associated with clinical responses in previously conducted clinical trials of PPV (6,14), were substantially induced in a subset of the vaccinated patients. Toxicity of PPV mainly involved skin reactions at the injection sites, and no severe adverse events were observed. Based on the positive immune responses to vaccine antigens and the safety profile, PPV could be further investigated as one of the promising approaches for refractory BTC.

The most unique aspect of PPV is the 'personalized' selection of antigen peptides ideal for individual patients in consideration of the pre-existing host immunity prior to vaccination (5-7). In view of the heterogeneity and complexity of host immune responses against tumors, this approach appears to be more rational than vaccination with non-personalized 'universal' tumor antigens. Notably, in the present study, the number of selected and vaccinated peptides was significantly associated with OS in the multivariate analysis, suggesting that greater numbers of peptides would be required for better clinical responses, possibly due to the heterogeneity and complexity of host immune responses against tumors.

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.

Acknowledgements

This study was supported by grants from the Regional Innovation Cluster Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Sendai-Kousei Hospital, the Kurozumi Medical Foundation and the Osaka Cancer Research Foundation.

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Cancer Science
The official journal of the Japanese Cancer Association JCA

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

(Received September 2, 2011/Revised December 16, 2011/Accepted December 25, 2011/Accepted manuscript online February 9, 2012/Article first published online February 13, 2012)

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).

doi: 10.1111/j.1349-7006.2012.02202.x

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 BenchMark 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

doi: 10.1111/j.1349-7006.2012.02202.x

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.

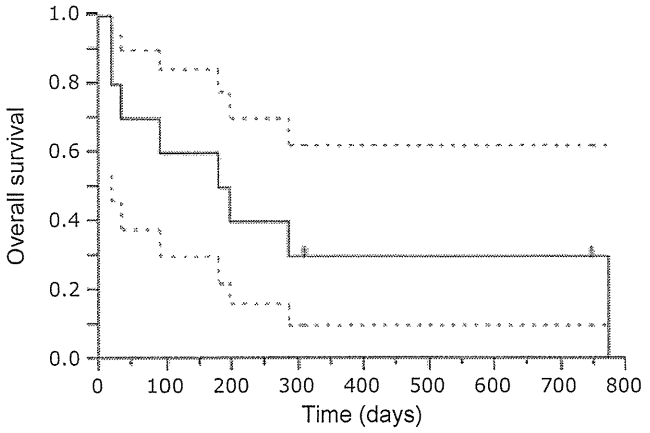

Fig. 1. Kaplan–Meier survival analysis in the enrolled patients. The median overall survival of patients who received personalized peptide vaccination (PPV) (n = 10; solid line) was 186.5 days and the 1 year survival rate was 30%. Dotted lines show 95% confidence intervals.

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

doi: 10.1111/j.1349-7006.2012.02202.x

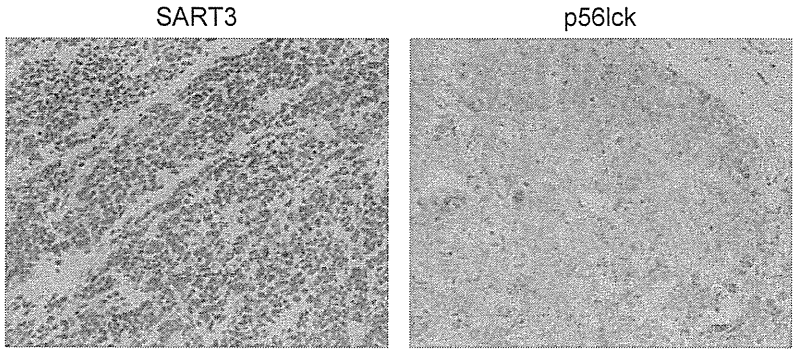

Fig. 2. Expression of the vaccine antigens in the tumor from a small cell lung cancer (SCLC) patient undergoing surgery after personalized peptide vaccination (PPV) treatment. The vaccine antigens SART3 and p56lck were detected by immunohistochemistry (IHC) with the antibodies specific to these molecules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

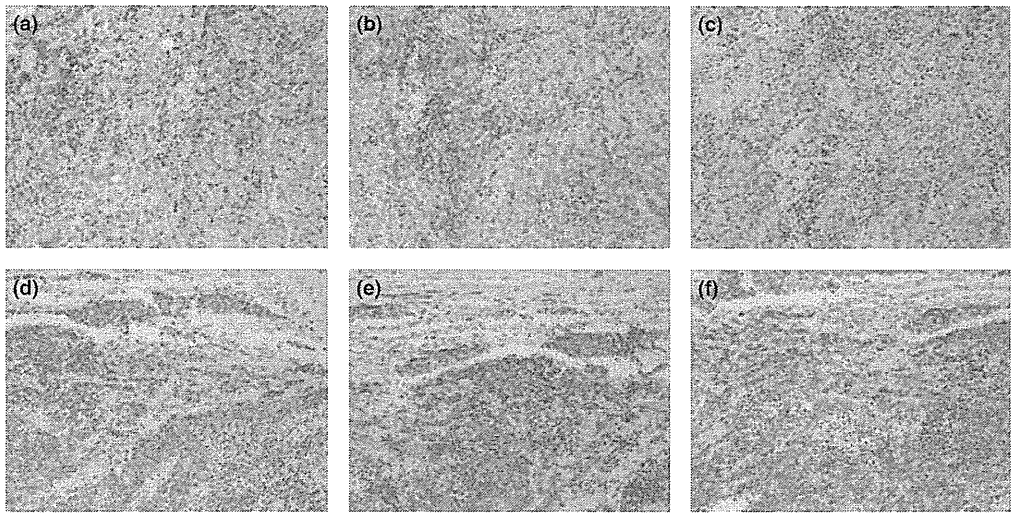

Fig. 3. Detection of tumor-infiltrating lymphocytes in tumors from small cell lung cancer (SCLC) patients treated with or without personalized peptide vaccination (PPV). Immune cells infiltrating within tumors were detected by immunohistochemistry (IHC) with the antibodies against CD3 (a and d), CD4 (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.

Acknowledgments

We thank Dr Shinji Tomimitsu (Shin Koga Hospital, Kurume, Japan) for preparing the tumor tissues from SCLC patients. This study was supported by the grants from the Regional Innovation Cluster Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan; Kurozumi Medical Foundation, and Osaka Cancer Research Foundation.

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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doi: 10.1111/j.1349-7006.2012.02202.x

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

AKIRA YAMADA[1], MASANORI NOGUCHI[2,4], NOBUKAZU KOMATSU[3], SHIGETAKA SUEKANE[4], SHIGERU YUTANI[3], FUKUKO MORIYA[4], TAKASHI MINE[3], KOSUKE MOMOZONO[4], KOICHIRO KAWANO[5] and KYOGO ITOH[3]

Divisions of [1]Cancer Vaccine Development, and [2]Clinical Research, Research Center for Innovative Cancer Therapy; Departments of [3]Immunology and Immunotherapy, [4]Urology, and [5]Obstetrics and Gynecology, Kurume University School of Medicine, Kurume, Fukuoka 830-0011, Japan

Received October 1, 2010; Accepted December 2, 2010

DOI: 10.3892/etm.2010.177

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

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

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

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

Introduction

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

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

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

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

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

Patients and methods

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

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

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

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

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

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

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

Table I. Vaccine candidate peptides.

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

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

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

Results

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

Table II. Patient characteristics.

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

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

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

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

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

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

Table III. Adverse events.

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

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

Discussion

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

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

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

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

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

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

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

Table V. Continued.

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

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

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

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

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

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

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

Acknowledgements

This study was supported in part by a grant from the 'High-Tech Research Center' Project for Private Universities, by matching fund subsidies from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, and by Grants-in-Aid from MEXT.

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Cancer Biology & Therapy 10:12, 1266-1279; December 15, 2010; © 2010 Landes Bioscience

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

Masanori Noguchi,[1,3,*] Takashi Mine,[4] Nobukazu Komatsu,[3] Shigetaka Suekane,[2] Fukuko Moriya,[2] Kei Matsuoka,[2] Shigeru Yutani,[3] Shigeki Shichijo,[3] Akira Yamada,[5] Uhi Toh,[6] Kouichiro Kawano,[7] Kouichi Azuma,[8] Hirotsugu Uemura,[9] Kiyotaka Okuno,[10] Kazumasa Matsumoto,[11] Hiroaki Yanagimoto,[12] Ryuya Yamanaka,[13] Masaaki Oka,[14] Satoru Todo,[15] Tetsuro Sasada[3] and Kyogo Itoh[3,5]

[1]Divisions of Clinical Research and [5]Cancer Vaccine; Innovative Research Center for Cancer Treatment; [2]Departments of Urology, [3]Immunology and Immunotherapy; [4]Multi-displenary Cancer Center; [6]Department of Surgery, [7]Gynecology, [8]Medicine; Kurume University School of Medicine; Kurume; Japan; [9]Department of Urology; Kinki University School of Medicine; Sakai; Japan; [10]Division of Colorectal Surgery; Department of Surgery; Kinki University School of Medicine; Osaka-Sayama; Japan; [11]Department of Urology; Kitasato University School of Medicine; Sagamihara; Japan; [12]Department of Surgery; Kansai Medical University; Hirakata; Japan; [1] [3]Brain Research Institute; Niigata University; Niigata; Japan; [14]Department of Digestive Surgery and Surgical Oncology; Yamaguchi University Graduate School of Medicine; Ube;Japan; [15]Department of General Surgery; Hokkaido University Graduate School of Medicine; Sapporo, Japan

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

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

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

Introduction

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

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

*Correspondence to: Masanori Noguchi; Email: noguchi@med.kurume-u.ac.jp
Submitted: 05/11/10; Revised: 07/17/10; Accepted: 08/29/10
DOI: 10.4161/cbt.10.12.13448