Table II. Characteristics of the enrolled patients.

Patient no.	Gender	Age (years)	PS	Disease type	Stage	Previous treatment (months)[a]	No. of vaccinations	Clinical response	OS (days)
1	M	59	0	ICC	R	GEM + S-1 (2)	18	SD	463
2	F	71	1	GBC	IVb	-	2	NA	57
3	F	59	1	GBC	IVb	GEM→GEM + CDDP (8)	4	NA	35
4	M	57	0	ECC	IVb	GEM + S-1 (3)	7	NA	116
5	M	75	0	GBC	IVb	GEM→GEM + S-1 (2)	5	NA	122
6	M	55	0	PAC	R	S-1→GEM (12)	14	SD	234
7	M	65	0	ECC	R	GEM→GEM + S-1 (4)	6	NA	102
8	M	73	1	ECC	R	GEM→S-1 (27)	3	NA	51
9	F	37	1	ECC	IVb	GEM + UFT→S-1 (7)	3	NA	48
10	F	69	0	ECC	R	GEM→S-1 (12)	24[b]	SD	455[c]
11	M	62	0	ECC	IVa	GEM→S-1 (6)	8	NA	177
12	M	49	0	GBC	R	GEM (6)	7	NA	111
13	F	56	0	ICC	R	-	16	SD	222
14	M	62	0	ECC	R	GEM + S-1(5)	12	PD	286
15	M	53	0	ICC	IVb	GEM (3)	6	SD	84
16	M	75	0	GBC	R	S-1 (2)	6	NA	292
17	M	79	0	ECC	IVb	S-1 (2)	12	NA	355[c]
18	M	59	0	ECC	IVb	GEM (2)	13	NA	207
19	F	56	0	GBC	IVb	GEM (2)	7	NA	92
20	M	71	0	ECC	R	GEM + S-1 (12)	11	NA	163[c]
21	M	51	0	ICC	R	GEM + S-1 (2)	12	SD	179[c]
22	M	66	0	ECC	IVa	GEM (3)	17[b]	SD	179[c]
23	M	52	1	ICC	IVa	5FU + CDDP→GEM + S-1 (14)	10	NA	101
24	M	41	0	ICC	IVa	GEM (4)	19[b]	PD	428[c]
25	F	48	0	GBC	IVa	-	14[b]	SD	125[c]

[a]Duration of previous chemotherapy; [b]under treatment; [c]patients alive. M, male; F, female; PS, performance status; ICC, intrahepatic cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma; GBC, gallbladder carcinoma; PAC, periampullary carcinoma; R, recurrent; GEM, gemcitabine; CDDP, cisplatin; UFT, tegafur-uracil; SD, stable disease; PD, progressive disease; OS, overall survival; NA, not assessed.

the PPV, 20 of 25 patients were treated in combination with chemotherapy, but the remaining 5 patients did not tolerate combined chemotherapy (patients 2, 9, 12, 13 and 25).

Of the 10 vaccinated patients whose radiological findings were available prior to and following the first cycle of vaccination, none had a complete response (CR) or partial response (PR). The best response was stable disease (SD) in 8 (80%) patients. The remaining 2 patients (20%) had progressive disease (PD) (Table II).

Toxicities. The overall toxicities are shown in Table III. The most frequent adverse events were dermatological reactions at the injection sites (n=17), hematological toxicity (n=14) and cholangitis (n=11). Severe adverse events (grade 3) were as follows: injection site reaction (n=1), gastrointestinal hemorrhage (n=2), gastrointestinal stricture (n=1), cholangitis (n=11), anemia (n=1), hyperbilirubinemia (n=1) and elevation of ALT (n=1) and ALP (n=1). According to an assessment by the independent safety evaluation committee in this trial, all of these severe adverse events, except for 1 case with a grade 3 injection site reaction, were due to cancer progression or other causes, rather than to the vaccinations themselves.

Immune responses to the vaccine peptides. Both humoral and T cell responses specific to the vaccine peptides were analyzed in blood samples prior to and following vaccination (data not shown). Plasma samples were obtained from 25, 20 and 8 patients before and at the end of the first (6th vaccination) and second (12th vaccination) cycles of vaccination, respectively. The post-vaccination samples were not available in the patients who failed to complete the first or second cycle of 6 vaccinations due to disease progression. The IgG responses specific to at least one of the vaccine peptides were augmented in 7 of 20 patients (35%) and in 7 of 8 patients (88%) at the end of the first and second cycles of vaccination, respectively.

T cell responses to the vaccine peptides were measured by IFN-γ ELISPOT assay with PBMCs. PBMCs were available for this assay in 22, 17 and 7 patients prior to and at the end of the first and second cycle of vaccination, respectively. In the pre-vaccination samples, antigen-specific T cell responses were detectable in 5 patients (23%). Of the 17 patients who completed the first cycle of vaccination, 8 patients (47%) showed an induction of T cell responses to the vaccine peptides. At the end of the second cycle of vaccination, the antigen-specific T cell responses were induced in 4 of 7 patients (57%). It

Table III. Toxicities.

	Grade 1	Grade 2	Grade 3	Total
Injection site reaction	11	5	1	17
Gastrointestinal (GI)				
GI hemorrhage	0	0	2	2
GI stricture	0	0	1	1
Abdominal distension	0	1	0	1
Constipation	0	1	0	1
Ascites	1	0	0	1
Hepatobiliary				
Cholangitis	0	0	11	11
Pulmonary				
Pleural effusion	1	0	0	1
Cardiac general				
Hypertension	0	1	0	1
Blood/bone marrow				
Anemia	9	1	1	11
Leukocytopenia	1	0	0	1
Lymphopenia	2	0	0	2
Laboratory				
Hyperbilirubinemia	1	0	1	2
AST elevation	4	1	0	5
ALT elevation	1	1	1	3
ALP elevation	3	2	1	6
Hypoalbuminemia	4	3	0	7
Hyperglycemia	0	3	0	3
Hyponatremia	1	0	0	1
Hypokalemia	0	1	0	1
Hypercalcemia	1	1	0	2
Creatinine elevation	1	0	0	1

should be noted that 3 of the 4 patients with positive T cell responses at the end of the second cycle of vaccination showed reactivity to more than 2 peptides. Collectively, substantial increases in peptide-specific IgG titers and/or T cell responses following vaccination were observed in a subset of the vaccinated patients.

Cytokines and inflammation markers. We then measured several cytokines, including IL-2, IL-4, IL-5, IL-6, IFN-γ and the inflammation markers, CRP and SSA, in the plasma prior to and following the first cycle of vaccination. IL-6 was detectable in 17 of 25 patients (68%) prior to vaccination (median, 2 pg/ml; range, 0-21). Among the 20 plasma samples available at the end of the first cycle of vaccination, IL-6 levels were increased, decreased or unchanged in 12, 5 or 3 patients, respectively (median 3 pg/ml; range 0-43). There was no significant difference in the levels of IL-6 between pre- and post-vaccination samples (P=0.118, 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).

The inflammation marker, CRP, was detectable in pre-vaccination plasma from all (100%) of the patients (median, 6.377 μg/ml; range, 0.043-8.891). Among the 20 plasma samples tested at the end of the first cycle of vaccination, plasma CRP levels were increased or decreased in 12 or 8 patients, respectively (median, 6.232 μg/ml; range, 1.331-17.332). Another inflammation marker, SAA, was also detected in pre-vaccination plasma from 21 (84%) of 25 patients (median, 113.486 μg/ml; range, 0-134.425). At the end of the first cycle of vaccination, plasma SAA levels were increased, decreased or unchanged in 12, 7 or 1 patients, respectively (median, 104.861 μg/ml; range, 0-138.917). There were no significant differences in the levels of CRP and SAA between pre- and post-vaccination samples (P=0.290 and P=0.252, respectively, Wilcoxon test).

Relationship between pre-vaccination clinical findings or laboratory data and OS. To identify potential biomarkers useful for selecting suitable patients for PPV, a Cox proportional hazards regression model was used with pre-vaccination clinical findings or laboratory data (Table IV). In the univariate analysis, IL-6, CRP, albumin, SAA and hemoglobin in pre-vaccination samples (P=0.002, P=0.004, P=0.008, P=0.031 and P=0.039, respectively), and the numbers of peptides selected for vaccination (P=0.039) were prognostic factors of OS. None of the other factors examined, such as age, gender, duration of previous chemotherapy, lymphocyte counts or frequencies of suppressive immune cell subsets (Treg and MDSCs) prior to vaccination, were statistically correlated with OS. Furthermore, multivariate Cox regression analysis was performed to define the clinical and laboratory features that were independently associated with OS by adjusting for possible confounding factors. Only the factors with a prognostic association in the univariate analysis, including IL-6, CRP, albumin, hemoglobin and the numbers of peptides selected for vaccination, were used for the multivariate analysis. SAA was not included for this analysis, since the levels of SAA were highly correlated with those of CRP (Pearson's correlation co-efficient 0.707; P=0.0002). As shown in Table IV, lower IL-6 and higher albumin levels in pre-vaccination samples and greater numbers of antigen peptides selected for vaccination were significantly favorable factors for OS [hazard ratio (HR) = 1.123, 95% confidence interval (CI) 1.008-1.252, P=0.035; HR=0.158, 95% CI 0.029-0.860, P=0.033; HR=0.258, 95% CI 0.098-0.682, P=0.006; respectively]. However, the other factors had no significant association.

Relationship between post-vaccination clinical findings or laboratory data and OS. To further identify potential post-vaccination markers for predicting patient prognosis, the univariate and multivariate Cox analyses were also carried out with post-vaccination clinical findings or laboratory data from the patients who completed the first cycle of 6 vaccinations (n=20). In the univariate analysis, levels of albumin, IL-6, CRP and hemoglobin (P=0.003, P=0.005, P=0.027 and P=0.031, respectively) and the number of vaccine peptides (P=0.033) were prognostic of OS. In addition, although not statistically significant, positive humoral responses to the vaccine peptides had a tendency to be associated with OS (P=0.089) and were also used for the multivariate Cox analysis. The multivariate analysis demonstrated that, among these factors with a potentially prognostic association in the univariate analysis, lower IL-6 levels and greater numbers of vaccine

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.

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

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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

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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 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

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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.

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.

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).

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.

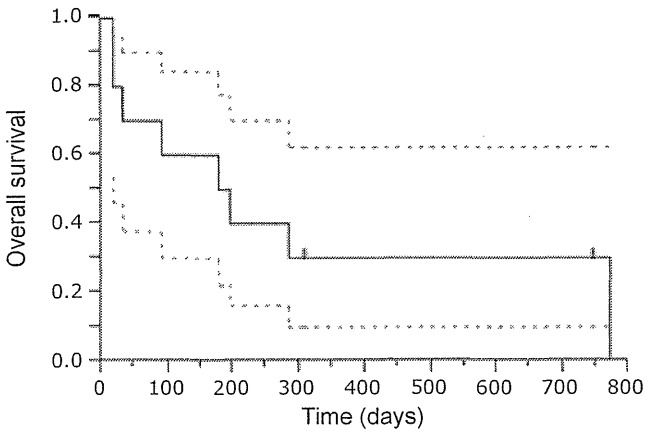

Fig. 1. Kaplan–Meier survival analysis in the enrolled patients. The median overall survival of patients who received personalized peptide vaccination (PPV) (n = 10; solid line) was 186.5 days and the 1 year survival rate was 30%. Dotted lines show 95% confidence intervals.

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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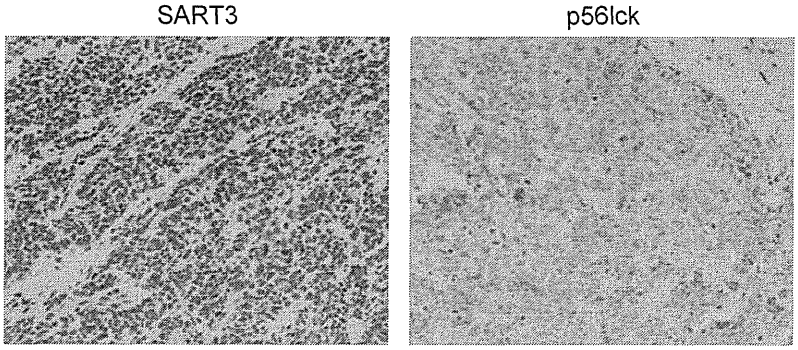

Fig. 2. Expression of the vaccine antigens in the tumor from a small cell lung cancer (SCLC) patient undergoing surgery after personalized peptide vaccination (PPV) treatment. The vaccine antigens SART3 and p56lck were detected by immunohistochemistry (IHC) with the antibodies specific to these molecules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

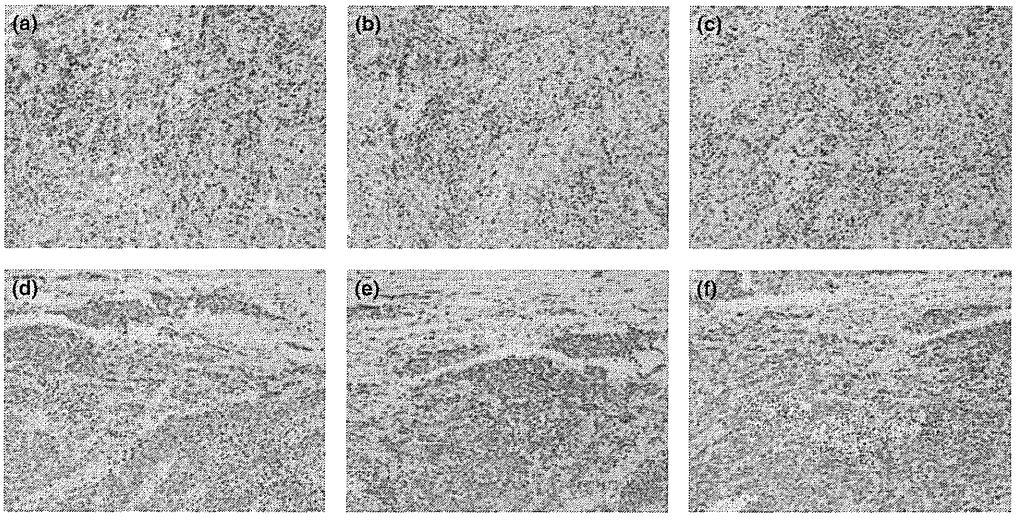

Fig. 3. Detection of tumor-infiltrating lymphocytes in tumors from small cell lung cancer (SCLC) patients treated with or without personalized peptide vaccination (PPV). Immune cells infiltrating within tumors were detected by immunohistochemistry (IHC) with the antibodies against CD3 (a and d), CD4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

Gene Expression Profiles in Peripheral Blood as a Biomarker in Cancer Patients Receiving Peptide Vaccination

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BACKGROUND: Because only a subset of patients show clinical responses to peptide-based cancer vaccination, it is critical to identify biomarkers for selecting patients who would most likely benefit from this treatment. **METHODS:** The authors characterized the gene expression profiles in peripheral blood of vaccinated patients to identify biomarkers to predict patient prognosis. Peripheral blood was obtained from advanced castration-resistant prostate cancer patients, who survived for >900 days (long-term survivors, n = 20) or died within 300 days (short-term survivors, n = 20) after treatment with personalized peptide vaccination. Gene expression profiles in prevaccination and postvaccination peripheral blood mononuclear cells (PBMCs) were assessed by DNA microarray. **RESULTS:** There were no statistically significant differences in the clinical or pathological features between the 2 groups. Microarray analysis of prevaccination PBMCs identified 19 genes that were differentially expressed between the short-term and long-term survivors. Among the 15 up-regulated genes in the short-term survivors, 13 genes, which were also differentially expressed in postvaccination PBMCs, were associated with gene signatures of granulocytes. When a set of 4 differentially expressed genes were selected as the best combination to determine patient survival, prognosis was correctly predicted in 12 of 13 patients in a validation set (accuracy, 92%). **CONCLUSIONS:** These results suggested that abnormal granulocytes present in the PBMC faction may contribute to poor prognosis in advanced prostate cancer patients receiving personalized peptide vaccination. Gene expression profiling in peripheral blood might thus be informative for devising better therapeutic strategies by predicting patient prognosis after cancer vaccines. ***Cancer* 2012;118:3208-21.** © *2011 American Cancer Society.*

KEYWORDS: peptide vaccine, peripheral blood, biomarker, microarray, granulocyte, interleukin 6.

INTRODUCTION

Together with the progressive increase of basic knowledge in tumor immunology, the field of cancer vaccines has dramatically moved forward.[1-5] However, because only a limited number of patients show clinically beneficial responses to cancer vaccination, it would be critical to identify clinical and/or biological markers useful for selecting patients who would most likely benefit from this treatment.[5-8] Recently, polymorphisms of several genes, including *CCR5*, interferon *(IFN)*-γ, interleukin *(IL)-6*, and cytotoxic T lymphocyte antigen 4 *(CTLA-4)*, have been reported to be associated with clinical responses in nonspecific immunotherapies, such as IL-2, IFN-α, Bacille Calmette-Guérin, and anti-CTLA-4 antibody therapies.[9-12] In addition, levels of serum cytokines or growth factors, including IL-1β, IL-1α, IL-6, tumor necrosis factor (TNF)-α, CCL3, CCL4, and vascular endothelial growth factor (VEGF), have also been shown to be correlated with clinical responses in nonspecific cytokine therapies.[13,14] However, because no reliable markers are currently in widespread use for predicting clinical outcomes in specific immunotherapies, novel biomarkers remain to be identified.

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Recently, high-throughput technologies have been developed as a novel approach to discovering biomarkers. In particular, DNA microarray technology is among the most widely recognized and extensively studied to identify new biomarkers for individualized therapies.[15-20] For example, gene expression profiles examined on a genome-wide scale in tumor tissues have been reported to clearly reflect clinical outcomes and/or responses to treatments in cancer patients.[15-17] In addition, expression array data of peripheral blood have also been shown to afford a comprehensive view of the patients' immune status in a variety of fields, including organ transplantation and autoimmune diseases.[18-20] However, there is little information available regarding gene expression profiles in peripheral blood of patients receiving cancer vaccines.

We have developed personalized peptide vaccination as a novel modality for cancer treatment, in which vaccine antigens are selected on the basis of pre-existing immune responses against vaccine antigens.[5,21-24] For example, our results in a recent small randomized clinical trial showed a potential clinical benefit of personalized peptide vaccination in advanced castration-resistant prostate cancer patients.[22] However, for further development of this approach, novel predictive biomarkers for selecting suitable patients with better clinical responses remain to be identified. Sipuleucel-T (Provenge; Dendreon Corporation, Seattle, Wash), an autologous active cellular immunotherapy product designed to stimulate a T-cell immune response against human prostatic acid phosphatase, was first approved for castration-resistant prostate cancer patients by the US Food and Drug Administration in 2010.[3] In this immunotherapy, CD54 up-regulation, a measure of the product's potency, has been reported to be correlated with patient overall survival.[25] However, this surrogate marker may be applicable only for dendritic cell-based immunotherapies. In the current study, we performed a gene expression profiling in peripheral blood samples of castration-resistant prostate cancer patients, who showed good or poor prognosis after personalized peptide vaccination, to identify promising biomarkers that are predictive of patient prognosis after treatment. Although it is likely that tumor tissues may have more informative gene signatures than peripheral blood mononuclear cells (PBMCs), they are usually difficult to obtain in patients with advanced castration-resistant prostate cancer. Therefore, given the ease of sampling and the ability to perform analyses at multiple time points, we used PBMCs for gene expression profiling in the current study. Our results suggested that the gene expression profiles in prevaccination PBMCs would be informative for devising better therapeutic strategies by predicting the subpopulation of castration-resistant prostate cancer patients who would most likely benefit from cancer vaccines.

MATERIALS AND METHODS

Patients

This is a retrospective analysis with peripheral blood samples from a subset of 164 patients with metastatic castration-resistant prostate cancer, who were positive for human leukocyte antigen (HLA)-A24 or HLA-A2 and enrolled in phase 1, 1-2, and 2 clinical trials for personalized peptide vaccination between February 2001 and April 2008.[22,24] These studies were approved by the ethics review committee at the participating hospitals in Japan (Kurume University Hospital, Kinki University Hospital, Okayama University Hospital, and Nara Medical University Hospital). Before enrollment in the studies, the history of all patients was studied, and physical examination, assessment of performance status, complete blood counts, serum biochemistry profiles, serum prostate-specific antigen (PSA) levels, chest radiograph, electrocardiogram, bone scan, and computerized tomography scans of the abdomen and pelvis were performed. Patients with a progression of disease (PD) after androgen ablation and second-line hormone therapy were enrolled. PD was defined by at least 1 of the following 3 criteria: 1) 2 consecutive 25% increases in PSA levels at least 2 weeks apart, 2) an increase of >25% in bidimensionally measurable soft tissue metastases, or 3) appearance of new foci on radionuclide bone scans. Other eligibility criteria included Eastern Cooperative Oncology Group performance status of 0 or 1, age of 18 years or more, normal hematologic, hepatic, and renal functions, and negative results on serologic tests for hepatitis B and hepatitis C. Patients with evidence of serious illness, an active secondary malignancy that occurred within 5 years before entry, or autoimmune diseases were excluded from the studies. After full explanation of the protocol, written informed consent was obtained from all patients before enrollment.

The right peptides for vaccination to individual patients were selected in consideration of the pre-existing host immunity before vaccination, assessed by titers of immunoglobulin (Ig)G specific to each of the 26 different vaccine candidates, as reported previously.[5,21-24] Peptides selected based on the results of peptide-specific IgG titers (3 or 4 peptides/vaccination; 3 mg/each peptide) were subcutaneously administrated with incomplete Freund

adjuvant (Montanide ISA51; Seppic, Paris, France) once per week for 6 consecutive weeks. After the first cycle of 6 vaccinations, antigen peptides, which were reselected according to the titers of peptide-specific IgG at every cycle of 6 vaccinations, were administered every 2 weeks while patients were allowed to continue the vaccinations.

Among the 164 patients enrolled, the patients who survived for >900 days (long-term survivors, n = 20) or who died within 300 days (short-term survivors, n = 20) were selected for analyses of gene expression profiles in PBMCs and soluble factors in plasma. The short-term and long-term survivors were defined in reference to a randomized, nonblinded, multinational phase 3 study of docetaxel-based regimens, TAX327, which involved 1006 men with castration-resistant prostate cancer,[26,27] because the disease conditions of castration-resistant prostate cancer patients in the TAX327 study were similar to those in the current study. Because each patient subgroup in the TAX327 study showed a median survival of 16.3 to 19.2 months,[28] we selected the patients who survived for >30 months (900 days) and who died within 10 months (300 days) as the long-term and short-term survivors, respectively, in the current study.

Blood Samples

PBMCs and plasma were used for measurement of gene expression profiles and soluble factors, respectively. Because this was a retrospective study with limited availability of patient samples, PBMCs or plasma from the patients were not equally available for each assay. Prevaccination PBMCs were analyzed by DNA microarray in all of the 40 selected patients (long-term survivors, n = 20; short-term survivors, n = 20). However, postvaccination PBMCs, which were obtained after the completion of 1 cycle of 6 vaccinations, were analyzed by DNA microarray in only a subset of the patients (long-term survivors, n = 16; short-term survivors, n = 14), because of failure in the completion of 1 cycle of vaccinations or the poor quality of purified RNA. Among these 30 postvaccination PBMCs, only 24 (long-term survivors, n = 12; short-term survivors, n = 12) were used for the quantitative real-time polymerase chain reaction (qRT-PCR) assay. Prevaccination plasma samples for soluble factor measurements were used from 36 patients (long-term survivors, n = 18; short-term survivors, n = 18).

The prevaccination PBMCs from all 40 patients were used as a training set to generate a gene classifier to predict patient prognosis. In addition, prevaccination PBMCs from 13 new independent cancer patients, who survived for >600 days (n = 6) or who died within 300 days (n = 7) after personalized peptide vaccination, were used in a validation test.

RNA Isolation From PBMCs

PBMCs were prepared from 20 mL of peripheral blood by density gradient centrifugation using Ficoll-Paque (GE Healthcare Life Sciences, Uppsala, Sweden). All samples were cryopreserved until RNA extraction. Total RNA was isolated using TRIZOL LS reagent (Invitrogen, Carlsbad, Calif) and purified using RNeasy Mini Kit (Qiagen, Valencia, Calif), according to the manufacturer's instructions. Quality and integrity of the purified total RNA were confirmed using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, Calif) and Nanodrop ND-1000 (Thermo Fisher Scientific, Wilmington, Del).

DNA Microarray Analysis

RNA amplification, labeling, and hybridization on HumanWG-6 v3.0 Expression BeadChip (Illumina Bead Array; Illumina, San Diego, Calif) were performed according to the manufacturer's instructions. Microarray data were extracted using BeadStudio v3.0 software (Illumina) and were then preprocessed and normalized using a variance-stabilizing transformation and robust spline normalization, as implemented in the lumi Bioconductor package. To filter low confidence probes that might increase the false-positive rates in subsequent statistical analyses, probes that did not reach a detection level with a P value <.05 in 70% of all samples were discarded. Accordingly, of the 48,803 probes on the chips, 16,449 remained above the reliable detection level. To assess the differential gene expression between the long-term and short-term survivors, we used the fold-change ranking, together with the P values, using the Linear Models for Microarray Data (Limma) Bioconductor package.[29] To determine the fold-change in the gene expression of the samples from the long-term survivors versus those from the short-term survivors, we calculated the fold-change values using the following formula: $\log_2$ fold-change = $\log_2(S_S/S_L)$, where S_L represented the assay range for a target gene in the samples from the long-term survivors and S_S represented that from the short-term survivors. Because the gene chip used in the current study (Illumina HumanWG-6 v3.0 Expression BeadChip) contained 48,803 probes, which corresponded to 25,409 annotated genes, some genes had multiple different probes on the gene chip. Therefore, the genes with multiple probes might be repeatedly detected by different probes and identified at multiple times in the list of differentially expressed genes.

Table 1. Patient Characteristics (Postvaccination Analysis)

Characteristic	Short-Term Survivors, n = 14	Long-Term Survivors, n = 16	*P*
Age, y			
Median (range)	62 (50-81)	71.5 (54-78)	.109
ECOG performance status, No. [%]			
0	13 [93]	16 [100]	.467
1	1 [7]	0 [0]	
HLA typing, No. [%]			
A24	10 [71]	9 [56]	.709
A2	3 [21]	6 [38]	
A24 and A2	1 [7]	1 [6]	
PSA, ng/mL			
Median (range)	79 (2-222)	34.5 (2-330)	.308
Gleason score, No. [%]			
7	3 [21]	5 [31]	.714
8	6 [43]	8 [50]	
9	5 [36]	3 [19]	
Site of metastasis, No. [%]			
None	2 [14]	2 [13]	.888
Bone only	10 [71]	13 [81]	
Bone and lymph nodes	1 [7]	0 [0]	
Other organs	1 [7]	1 [6]	
Number of vaccinations			
Median (range)	9 (5-14)	52.5 (10-124)	<.001
Survival time, d			
Median {95% CI}	248.5 {176-277}	1482 {1120-1764}	<.001

Abbreviations: CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; HLA, human leukocyte antigen; PSA, prostate-specific antigen.

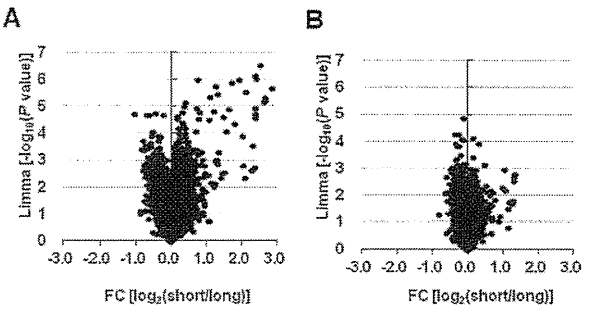

Figure 1. Volcano plots present the microarray data in prevaccination and postvaccination peripheral blood mononuclear cells (PBMCs). The plot graphs the fold-change (FC; $\log_2$[short/long]) on the x-axis versus statistical significance (minus $\log_{10}$ *P* value) on the y-axis in PBMCs (A) after and (B) before the peptide vaccines.

qRT-PCR

After the total RNA (200 ng) from postvaccination PBMCs (long-term survivors, n = 12; short-term survivors, n = 12) was reverse-transcribed into the first-strand cDNA with PrimeScript RT reagent kit (Takara Bio, Shiga, Japan), qRT-PCR was performed with a SYBR Premix Ex Taq II kit (Takara Bio) by using a Thermal Cycler Dice Real Time System (Takara Bio). The data were evaluated by the ddCT method. The number of copies of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was measured in each cDNA sample as an internal control. The expression of each gene was normalized to that of *GAPDH*. The sequences of the primers for qRT-PCR were as follows: defensin alpha 1 (*DEFA1*): forward, 5′-CGGACATCCCAGAAGTGGTTG-3′, reverse, 5′-CCCTGGTAGATGCAGGTTCCATA-3′; defensin alpha 4 (*DEFA4*): forward, 5′-CACTCCAGGCAAGAGGTGATGA-3′, reverse, 5′-GAGGCAGTTCCCAACACGAAGT-3′; myeloperoxidase (*MPO*): forward, 5′-CTGCATCATCGGTACCCAGTTC-3′, reverse, 5′-GATGCCTGTGTTGTCGCAGA-3′; carcinoembryonic antigen-related cell adhesion molecule 8 (*CEACAM8*): forward, 5′-TGGCACATTCCAGCAATACACA-3′, reverse, 5′-ATCATGATGCTGACAGTGGCTCTA-3′; *GAPDH*: forward, 5′-GCACCGTCA

Table 2. Differentially Expressed Genes in Postvaccination Peripheral Blood Mononuclear Cells

Gene Symbol	Gene Name	Fold-Change[a]	P[b]	Expression[c]	Before and After[d]
LTB	Lymphotoxin beta	−1.03	<.001		
OLR1	Oxidized low-density lipoprotein receptor 1	1.04	.004		
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	1.07	<.001	G	
ARG1	Arginase, liver	1.10	<.001	G	
MYL4	Myosin, light chain 4, alkali; atrial, embryonic	1.14	.007		
ALAS2	Delta-aminolevulinate, synthase 2	1.20	.009	E	
SLPI	Secretory leukocyte peptidase inhibitor	1.22	<.001	G	
SELENBP1	Selenium-binding protein 1	1.22	.008		
SNCA[e]	Synuclein, alpha	1.25	.008		
AZU1	Azurocidin 1	1.25	<.001	G	#
HMGXB4	HMG box domain containing 4	1.27	.001		
RNASE3	Ribonuclease, RNase A family, 3	1.28	.001	G	#
HBQ1	Hemoglobin, theta 1	1.31	.001	E	
MMP9	Matrix metallopeptidase 9	1.32	<.001	G	
GYPE	Glycophorin E	1.36	<.001	E	
SNCA[e]	Synuclein, alpha	1.39	.005		
EPB42	Erythrocyte membrane protein band 4.2	1.45	.003	E	
HP	Haptoglobin	1.50	<.001	E	
IFIT1L	Interferon-induced protein with tetratricopeptide repeats 1-like	1.51	.003		
CD24	CD24 molecule	1.55	<.001	G	
BPI	Bactericidal/permeability-increasing protein	1.64	<.001	G	
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6	1.72	<.001	G	#
PGLYRP1	Peptidoglycan recognition protein 1	1.80	<.001	G	#
MPO	Myeloperoxidase	1.94	<.001	G	#
OLFM4	Olfactomedin 4	2.01	<.001		
HBM	Hemoglobin, mu	2.05	.002	E	
ALAS2	Delta-aminolevulinate, synthase 2	2.11	.005	E	
CEACAM8	Carcinoembryonic antigen-related cell adhesion molecule 8	2.13	<.001	G	#
ERAF	Erythroid-associated factor	2.29	.002	E	
CA1	Carbonic anhydrase I	2.31	<.001	G	
HBD	Hemoglobin, delta	2.37	.002	E	
LCN2	Lipocalin 2	2.40	<.001	G	#
CTSG	Cathepsin G	2.40	<.001	G	#
DEFA1[e]	Defensin, alpha 1	2.40	<.001	G	#
CAMP	Cathelicidin antimicrobial peptide	2.41	<.001	G	#
ELA2	Elastase 2, neutrophil	2.44	<.001	G	#
DEFA4	Defensin, alpha 4, corticostatin	2.53	<.001	G	#
DEFA3	Defensin, alpha 3, neutrophil-specific	2.65	<.001	G	#
DEFA1[e]	Defensin, alpha 1	2.65	<.001	G	#
DEFA1[e]	Defensin, alpha 1	2.67	<.001	G	#
DEFA1[e]	Defensin, alpha 1	2.68	<.001	G	#
DEFA1[e]	Defensin, alpha 1	2.87	<.001	G	#

[a] $\log_2$ (short/long).
[b] Limma *P* value.
[c] Preferential expression in granulocyte (G) and erythroid cells (E).
[d] Commonly identified in both prevaccination and postvaccination peripheral blood mononuclear cells (#).
[e] Identified by multiple different probes on the gene chip.

AGGCTGAGAAC-3′, reverse, 5′-TGGTGAAGACGC CAGTGGA-3′.

Measurement of Soluble Factors in Plasma

To detect the plasma levels of cytokines, chemokines, and growth factors before vaccination (long-term survivors, n = 18; short-term survivors, n = 18), a bead-based multiplex assay (xMAP; Luminex, Austin, Tex) was used. For this assay, multiple soluble factors were measured in duplicate 100 μL aliquots of plasma by using the Luminex 200 system according to the manufacturer's instructions. The analyte kit used for the measurement of the levels of multiple cytokines, chemokines, and growth factors, including IL-1Rα, IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-α, IFN-γ, TNF-α, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-inducible protein (IP)-10,

RANTES, Eotaxin, macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemoattractant protein (MCP)-1, monokine induced by interferon-gamma (MIG), VEGF, endothelial growth factor (EGF), human growth factor (HGF), and basic fibroblast growth factor (FGF), was obtained from Invitrogen (Human 30-Plex).

Statistical Analysis

Mann-Whitney and Fisher exact tests were used for statistical analyses of clinical and pathological features of the patients. Overall survival was estimated by the Kaplan-Meier method and log-rank test. Mann-Whitney test was used to compare the plasma levels of cytokines, chemokines, and growth factors, and the gene expression levels in PBMCs assessed by qRT-PCR. All tests were 2-sided, and the differences with P values $<.05$ were considered statistically significant. In identification of differentially expressed genes in PBMCs, the data were assessed by the fold-change ranking, together with a nonstringent P value cutoff.[29] From the differentially expressed genes, the genes critical for accurate classification of the short-term and long-term survivors were selected by stepwise discriminant analysis method. The classification performance of the selected genes was validated in an independent test set (n = 13) by determining sensitivity, specificity, positive predictive value, negative predictive value, and accuracy. All statistical analyses were conducted using SAS version 9.1 (SAS Institute, Cary, NC).

RESULTS

Patients

We selected advanced castration-resistant prostate cancer patients who received personalized peptide vaccination and who survived for >900 days (long-term survivors, n = 20) or died within 300 days (short-term survivors, n = 20) for the DNA microarray analysis in PBMCs. For personalized peptide vaccination, different combinations of 4 peptides were selected based on the results of peptide-specific IgG titers in all patients, except for 1 patient receiving 3 peptides in the short-term survivors. Numbers of vaccinations were significantly different between the long-term (median, 50; range, 10-124) and short-term (median, 8; range, 3-14) survivors ($P < .001$). PSA doubling time calculated by the log-slope method in the long-term and short-term survivors after personalized peptide vaccination was negative in 10 (50%) of 20 and in 4 (20%) of 20 patients, respectively. In the remaining patients positive for PSA doubling time, the long-term survivors (median, 13.6; range 1.6-92.9; n = 10) had a longer PSA doubling time ($P = .006$) than the short-term survivors (median, 2.1; range, 0.7-79.0; n = 16).

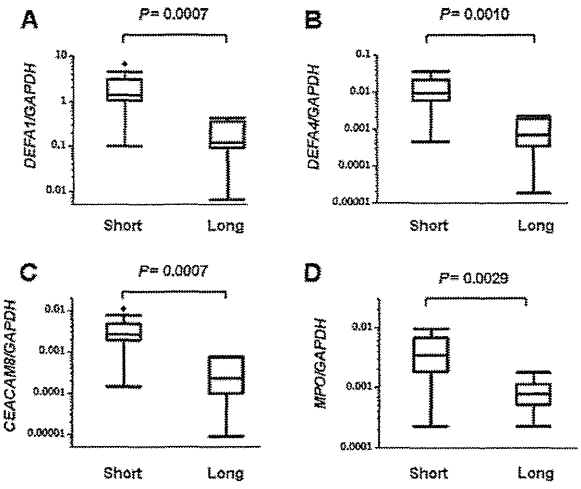

Figure 2. Differential gene expression was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). The gene expression of (A) *DEFA1*, (B) *DEFA4*, (C) *CEACAM8*, and (D) *MPO* were measured by qRT-PCR in postvaccination peripheral blood mononuclear cells of the short-term (n = 12) and long-term (n = 12) survivors. The expression of each gene was normalized to that of *GAPDH*. The expression ratios of each gene are shown. Box plots show median and interquartile range (IQR). The whiskers (vertical bars) are the lowest value within 1.5 × IQR of the lower quartile and the highest value within 1.5 × IQR of the upper quartile. Data not included between the whiskers were plotted as outliers with dots. Two-sided P values were calculated with Mann-Whitney test.

Identification of Differentially Expressed Genes in Postvaccination PBMCs

We first analyzed postvaccination PBMCs by using DNA microarray analysis (HumanWG-6 v3.0 Expression BeadChip; 48,803 probes corresponding to 25,409 genes in total) to determine the genes that were differentially expressed between the long-term and short-term survivors. As shown in Table 1, there were no statistically significant differences in the clinical or pathological features except for the number of vaccinations ($P < .001$) and overall survival (log-rank test, $P < .001$) between the long-term (n = 16) and short-term (n = 14) survivors in whom postvaccination PBMCs were analyzed. Figure 1A shows a volcano plot that graphs the $\log_2$ fold-change on the x-axis versus the statistical significance (negative $\log_{10}$ P value) on the y-axis. When the data were assessed by fold-change ranking ($\log_2$ fold-change <-1.0 or >1.0) together with P values ($P < .01$), expressions of 42 probes, corresponding to 38 genes, were significantly altered

Table 3. Patient Characteristics (Prevaccination Analysis)

Characteristic	Short-Term Survivors, n = 20	Long-Term Survivors, n = 20	*P*
Age, y			
Median (range);	62 (50-81)	71 (54-78)	.058
ECOG performance status, No. [%]			
0	17 [85]	20 [100]	.231
1	3 [15]	0 [0]	
HLA typing, No. [%]			
A24	13 [65]	12 [60]	1.000
A2	5 [25]	6 [30]	
A24 and A2	2 [10]	2 [10]	
PSA, ng/mL			
Median (range)	73.5 (2-296)	34.5 (2-330)	.239
Gleason score, No. [%]			
7	4 [20]	5 [25]	.710
8	8 [40]	10 [50]	
9	8 [40]	5 [25]	
Site of metastasis, No. [%]			
None	2 [10]	3 [15]	1.000
Bone only	14 [70]	14 [70]	
Bone and lymph nodes	3 [15]	2 [10]	
Other organs	1 [5]	1 [5]	
Number of vaccinations			
Median (range)	8 (3-14)	50 (10-124)	<.001
Survival time, d			
Median {95% CI}	196 {135-273}	1482 {1120-1764}	<.001

Abbreviations: CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; HLA, human leukocyte antigen; PSA, prostate-specific antigen.

between the 2 groups; 1 gene was down-regulated, whereas the remaining 37 were up-regulated in the short-term survivors (Table 2). Notably, 20 of the 37 up-regulated genes are known to be preferentially expressed in granulocytes. For example, many of them, including defensins (*DEFA1, DEFA3, DEFA4), ELA2, CTSG, CAMP,* and *MPO*, are reportedly localized within the granules in granulocytes and related to defense responses. In addition, other granulocyte-related molecules, such as matrix metalloproteinase 9 (*MMP9*) and arginase-1 (*ARG1*), are known to play important roles in tumor promotion and immune suppression.[30,31] The differential gene expression detected by the microarray analysis was further confirmed by qRT-PCR for some of the identified genes, including *DEFA1, DEFA4, CEACAM8*, and *MPO* (Fig. 2).

Identification of Differentially Expressed Genes in Prevaccination PBMCs

We next investigated the differentially expressed genes in prevaccination PBMCs from the long-term and short-term survivors. There were no statistically significant differences in the clinical or pathological features except for the number of vaccinations ($P < .001$) and overall survival (log-rank test, $P < .001$) between the long-term (n = 20) and short-term (n = 20) survivors in whom prevaccination PBMCs were analyzed (Table 3). As shown in the volcano plot, both fold-change and Limma *P* values in prevaccination samples were substantially lower than those in the postvaccination samples (Fig. 1B). Indeed, when the data were assessed with the same criteria as those for the postvaccination samples ($\log_2$ fold-change <-1.0 or >1.0 and $P < .01$), only 5 genes (5 probes) were identified as being differentially expressed (data not shown). However, when a less stringent criterion ($\log_2$ fold-change <-0.6 or >0.6 and $P < .05$) was used, 19 genes (23 probes) were identified; among these, 4 genes were down-regulated, whereas 15 were up-regulated in the short-term survivors (Table 4). Notably, of the 15 up-regulated genes, 13 genes, all of which were commonly identified in both prevaccination and postvaccination PBMCs, were associated with gene signatures of granulocytes.

Table 4. Differentially Expressed Genes in Prevaccination Peripheral Blood Mononuclear Cells

Gene Symbol	Gene Name	Fold-Change[a]	P[b]	Expression[c]	Before and After[d]
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha	−0.82	.049		
LRRN3	Leucine-rich repeat neuronal 3	−0.61	.008		
PCDH17	Protocadherin 17	−0.60	.002		
TTN	Titin	−0.60	.008		
LAIR2	Leukocyte-associated immunoglobulin-like receptor 2	0.60	.032		
RNASE3	Ribonuclease, RNase A family, 3	0.63	.020	G	#
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6	0.65	.010	G	#
AZU1	Azurocidin 1	0.66	.006	G	#
HIST1H4C	Histone cluster 1, H4c	0.71	.025		
PGLYRP1	Peptidoglycan recognition protein 1	0.72	.007	G	#
CEACAM8	Carcinoembryonic antigen-related cell adhesion molecule 8	0.78	.015	G	#
LCN2	Lipocalin 2	1.00	.005	G	#
MPO	Myeloperoxidase	1.04	.001	G	#
CAMP	Cathelicidin antimicrobial peptide	1.09	.007	G	#
DEFA1[e]	Defensin, alpha 1	1.17	.031	G	#
DEFA1[e]	Defensin, alpha 1	1.20	.018	G	#
DEFA1[e]	Defensin, alpha 1	1.26	.018	G	#
DEFA3	Defensin, alpha 3, neutrophil-specific	1.27	.017	G	#
DEFA1[e]	Defensin, alpha 1	1.27	.020	G	#
DEFA1[e]	Defensin, alpha 1	1.30	.015	G	#
CTSG	Cathepsin G	1.32	.003	G	#
DEFA4	Defensin, alpha 4, corticostatin	1.33	.002	G	#
ELA2	Elastase 2, neutrophil	1.36	.002	G	#

[a] Log_2 (short/long).
[b] Limma *P* value.
[c] Preferential expression in granulocyte (G).
[d] Commonly identified in both prevaccination and postvaccination peripheral blood mononuclear cells (#).
[e] Identified by multiple different probes on the gene chip.

Changes in the Gene Expression Profiles in PBMCs After Personalized Peptide Vaccination

To investigate how personalized peptide vaccination affected the gene expression profiles in PBMCs, we further compared them between before and after personalized peptide vaccination in the long-term (n = 16) and short-term survivors (n = 14). The changes were assessed by fold-change ranking (log_2 fold-change <-1.0 or >1.0) together with *P* values ($P < .01$). In the long-term survivors, only 1 gene, titin (*TTN*), was down-regulated (log_2 fold-change $= -1.04$, $P < .001$) after personalized peptide vaccination, whereas no genes were up-regulated. In contrast, as shown in Table 5, 41 genes (47 probes) were up-regulated after personalized peptide vaccination, whereas no genes were down-regulated in the short-term survivors. Notably, many of the 41 up-regulated genes in the short-term survivors were also identified as being differentially expressed in pre- and/or postvaccination PBMCs.

Selection of a Gene Classifier for Predicting Patient Prognosis After Personalized Peptide Vaccination

One of the most important applications of microarray-based gene expression data is the ability to predict clinical endpoints after treatments.[18-20] Thus, we examined whether the gene expression profile obtained by DNA microarray analysis of prevaccination PBMCs would be useful for predicting patient prognosis after personalized peptide vaccination. When a stepwise discriminant analysis method was used to choose a gene set from the 23 probes differentially expressed in the prevaccination PBMCs, a combination of 4 genes, *LRRN3, PCDH17, HIST1H4C,* and *PGLYRP1*, gave the best prediction of short-term survivors, with a sensitivity, specificity,

Table 5. Upregulated Genes After Vaccination in Peripheral Blood Mononuclear Cells From the Short-Term Survivors

Gene Symbol	Gene Name	Fold-Change[a]	*P*[b]	Expression[c]	Before and After[d]
RNASE2	Ribonuclease, RNase A family, 2	1.02	<.001		
SLC4A1	Solute carrier family 4, anion exchanger, member 1	1.06	.008	E	
HEMGN	Hemogen (HEMGN), transcript variant 2	1.08	.001	E	
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	1.09	<.001	G	After
S100P	S100 calcium-binding protein P	1.09	.001		
ALS2	Amyotrophic lateral sclerosis 2	1.09	.001		
ARG1	Arginase, liver	1.10	<.001	G	After
SLPI	Secretory leukocyte peptidase inhibitor	1.12	<.001	G	After
OLR1	Oxidized low-density lipoprotein (lectin-like) receptor 1	1.14	<.001		After
RETN	Resistin	1.15	.005		
HBQ1	Hemoglobin, theta 1	1.16	.007	E	After
ALAS2[e]	Delta-aminolevulinate, synthase 2	1.19	.004	E	After
MMP9	Matrix metallopeptidase 9	1.22	<.001	G	After
RNASE3	Ribonuclease, RNase A family, 3	1.24	<.001	G	Before, after
HMGXB4	HMG box domain containing 4	1.24	.003		After
SELENBP1	Selenium-binding protein 1	1.24	.003		After
GYPE	Glycophorin E	1.36	.001	E	After
BPI	Bactericidal/permeability-increasing protein	1.36	<.001	G	After
TCN1	Transcobalamin I	1.38	<.001	G	
ORM1	Orosomucoid 1	1.38	<.001		
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6	1.40	<.001	G	Before, after
SNCA[e]	Synuclein, alpha	1.40	.001		After
MPO	Myeloperoxidase	1.44	.002	G	Before, after
SNCA[e]	Synuclein, alpha	1.44	<.001		After
HP	Haptoglobin	1.46	<.001	E	After
CD24	CD24 molecule	1.48	<.001	G	After
IFIT1L	Interferon-induced protein with tetratricopeptide repeats 1-like	1.55	.003		After
EPB42	Erythrocyte membrane protein band 4.2	1.56	.002	E	After
CTSG	Cathepsin G	1.56	.004	G	Before, after
ELA2	Elastase 2, neutrophil	1.74	.002	G	Before, after
PGLYRP1	Peptidoglycan recognition protein 1	1.77	<.001	G	Before, after
DEFA1[e]	Defensin, alpha 1	1.79	<.001	G	Before, after
CEACAM8	Carcinoembryonic antigen-related cell adhesion molecule 8	1.80	<.001	G	Before, after
HBM	Hemoglobin, mu	1.86	.005	E	After
DEFA4	Defensin, alpha 4, corticostatin	1.91	<.001	G	Before, after
ALAS2[e]	Delta-aminolevulinate, synthase 2	1.94	.005	E	After
CAMP	Cathelicidin antimicrobial peptide	2.03	<.001	G	Before, after
LCN2	Lipocalin 2	2.04	<.001	G	Before, after
OLFM4	Olfactomedin 4	2.05	<.001		After
DEFA3	Defensin, alpha 3, neutrophil-specific	2.12	<.001	G	Before, after
DEFA1[e]	Defensin, alpha 1	2.12	<.001	G	Before, after
DEFA1[e]	Defensin, alpha 1	2.16	<.001	G	Before, after
DEFA1[e]	Defensin, alpha 1	2.25	<.001	G	Before, after
ERAF	Erythroid associated factor	2.29	.002	E	After
CA1	Carbonic anhydrase I	2.45	<.001	G	After
HBD	Hemoglobin, delta	2.48	.001	E	After
DEFA1[e]	Defensin, alpha 1	2.73	<.001	G	Before, after

[a] $\log_2$ (postvaccination/prevaccination).
[b] Limma *P* value.
[c] Preferential expression in granulocytes (G) and erythroid cells (E).
[d] Identified as differentially expressed genes in prevaccination and/or postvaccination peripheral blood mononuclear cells.
[e] Identified by multiple different probes on the gene chip.

positive predictive value, negative predictive value, and accuracy of 85%, 75%, 77%, 83%, and 80%, respectively (Table 6). Importantly, when this 4-gene classifier was used in 13 new independent cancer patients as a validation test, prognosis was correctly predicted in 12 of the 13 patients with a sensitivity, specificity, positive predictive