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

Table 6. Selection of a Gene Classifier for Predicting Short-Term Survival

Training/Test	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Accuracy (%)
Training, n = 40	17/20 (85)	15/20 (75)	17/22 (77)	15/18 (83)	32/40 (80)
Test, n = 13	7/7 (100)	5/6 (83)	7/8 (88)	5/5 (100)	12/13 (92)

value, negative predictive value, and accuracy of 100%, 83%, 88%, 100%, and 92%, respectively, for the prediction of short-term survival (Table 6).

Increase in the Prevaccination Plasma IL-6 Levels in the Patients With Poor Prognosis

Expression of cytokines, chemokines, and growth factors, which may result from proinflammatory and/or anti-inflammatory tumor microenvironments, gives a broad picture of the immunological status of cancer patients.[32-35] We therefore examined the levels of these soluble factors using a bead-based multiplex assay with prevaccination plasma samples from the long-term and short-term survivors. As shown in Figure 3, the plasma levels of proinflammatory cytokine IL-6 were significantly higher in the short-term survivors than in the long-term survivors ($P =$ .009). However, the plasma levels of other cytokines, chemokines, or growth factors, including IL-1Rα, IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-α, IFN-γ, TNF-α, G-CSF, GM-CSF, IP-10, RANTES, Eotaxin, MIP-1α, MIP-1β, MCP-1, MIG, VEGF, EGF, HGF, and basic FGF, were not significantly different between the 2 groups (data not shown).

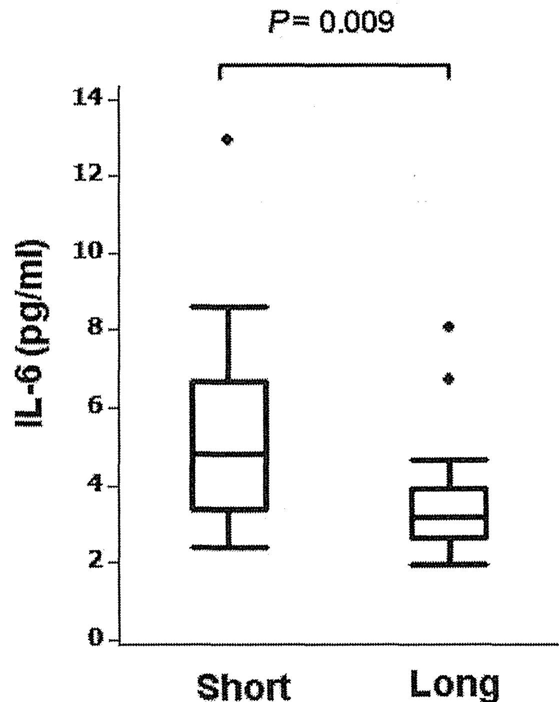

Figure 3. Increase in plasma interleukin (IL)-6 levels in the short-term survivors is shown. The levels of IL-6 assessed by bead-based multiplex assay in prevaccination plasma were compared between the short-term (n = 18) and long-term (n = 18) survivors. Box plots show median and interquartile range (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 value was calculated with Mann-Whitney test.

DISCUSSION

The identification of biomarkers to predict clinical responses to treatment is a challenging but important issue for the development of individualized therapies.[5-8] Although recent advances in high-throughput microarray technology have allowed gene expression profiling for subclassifications of patients in a variety of fields, including organ transplantation and autoimmune diseases,[18-20] little information is available regarding gene expression profiles in peripheral blood of patients treated with immunotherapies. In the current study, to identify promising biomarkers that are predictive of patient prognosis after personalized peptide vaccination, we examined gene expression profiles in PBMCs from 40 advanced castration-resistant prostate cancer patients who showed good or poor prognosis after personalized peptide vaccination. Our DNA microarray analysis in PBMCs identified distinctive genes that were differentially expressed between the long-term and short-term survivors. Interestingly, a statistical prediction model provided a 4-gene classifier that was able to predict patient prognosis with an accuracy of 92% in a validation test, suggesting that the identification of suitable patients for cancer vaccines may be possible with the profiling of a modest number of genes in peripheral blood samples. Because there were no significant differences in the other clinical and pathological

features of the patients enrolled in the current study, except for the number of vaccinations and overall survival, our findings seem to be quite informative for the further development of cancer vaccines.

In the current study, 4 genes, *LRRN3, PCDH17, HIST1H4C,* and *PGLYRP1*, were selected as the best combination for prediction of patient prognosis. *LRRN3* gene encodes a highly conserved transmembrane protein with multiple leucine-rich repeats, which is abundantly expressed in the developing and adult central nervous system. Polymorphisms in this gene were reported to be associated with autism spectrum disorder susceptibility.[36] *PCDH17* is 1 of the cadherin superfamily genes and is expressed predominantly in the nervous system. This molecule was reported to be a tumor suppressor gene candidate in squamous cell carcinomas.[37] *HIST1H4C* gene encodes a member of the histone H4 family, which forms the nucleosome structure of the chromosomal fiber, and may play a central role in transcription regulation, DNA repair and replication, and chromosomal stability.[38] *PGLYRP1* gene encodes a pattern recognition receptor related to innate immunity against bacteria, which is expressed primarily in the granules of granulocytes.[39] Although this information is available from the literature, little is known about the roles of these molecules in immune responses to cancer vaccines. Further studies remain to be done to elucidate them.

One of the most striking features of the differentially expressed genes is that many of the up-regulated genes in both prevaccination and postvaccination PBMCs from the short-term survivors were associated with gene signatures of granulocytes. This may possibly be reflected by the different frequencies of granulocytes in the PBMC fraction purified from peripheral whole blood on density gradient centrifugation using Ficoll-Paque. In healthy donors, normal granulocytes are usually separated from the PBMC fraction on Ficoll-Paque density gradient. However, patients with various types of cancers have been reported to show increased numbers of activated granulocytes in their peripheral blood, which are purified in the PBMC fraction.[40-42] Recently, these abnormal granulocytes have been defined as granulocytic myeloid-derived suppressor cells, which express higher levels of inhibitory molecules, such as ARG1 and inducible nitric oxide synthase,[41,42] and impair the immunological functions of T cells and other immune cells.[43-45] In addition, several studies have recently shown the critical roles for neutrophils, a main subset of granulocytes, in tumorigenesis.[46] Neutrophils have a significant impact on the tumor microenvironment by producing cytokines, chemokines, and other products, such as reactive oxygen species and proteinases, which regulate inflammatory cell activation/recruitment, tumor cell proliferation, angiogenesis, and metastasis.[47-49] For example, recent clinical studies have revealed that the presence of neutrophils in tumors was significantly associated with poor outcomes.[50,51] Unfortunately, because of the limited availability of blood samples, we have not fully characterized the granulocytes that were purified in the PBMC fraction, but it is highly possible that abnormal granulocytes in peripheral blood inhibit beneficial immune responses and lead to poor prognosis after peptide vaccines. The current study might provide a novel treatment approach capable of enhancing the clinical efficacy of cancer vaccines. Recently, chemotherapeutic drugs, such as gemcitabine and 5-fluorouracil, have been shown to selectively eliminate myeloid-derived suppressor cells in mice.[52,53] In addition, targeting of VEGF-mediated signaling using a tyrosine kinase inhibitor, sunitinib, has been reported to block expansion of $CD15^{+}CD14^{-}$ granulocytic myeloid-derived suppressor cells in patients with renal cell cancers.[54] It would thus be possible that accompanying treatments with such chemotherapeutic or molecularly targeted drugs before providing cancer vaccines suppress the gene signatures related to poor prognosis and improve patient outcomes after personalized peptide vaccination.

In addition to the granulocyte-related genes, other interesting genes were also differentially expressed between the long-term and short-term survivors. For example, leukocyte-associated immunoglobulin-like receptor 2 (*LAIR2*), a member of the immunoglobulin superfamily, was down-regulated in the prevaccination PBMCs of short-term survivors. Although not well studied, this molecule has been suggested to function as a proinflammatory mediator by suppressing the homologous immune inhibitor, leukocyte-associated immunoglobulin-like receptor 1 (*LAIR-1*), which is present on several types of mononuclear leukocytes.[55] In addition, another noticeable finding is that several erythroid-specific genes, such as hemoglobin families (*HBQ1, HBM, HBD), ALAS2, GYPE, EPB42, HP*, and *ERAF*, were up-regulated in the postvaccination PBMCs of short-term survivors. The precise roles of these differentially expressed genes in immune responses to cancer vaccines need to be determined.

Interestingly, when the gene expression profiles in PBMCs were compared between before and after personalized peptide vaccination, many of the differentially

expressed genes in prevaccination and/or postvaccination PBMCs, including granulocyte-related and erythroid-related genes, were up-regulated after personalized peptide vaccination in the short-term survivors, but not in the long-term survivors. This finding may be explained by the possibility that induction of granulocyte and erythroid gene signatures may be prevented by personalized peptide vaccination in the long-term survivors.

It should also be noted that the levels of the proinflammatory cytokine IL-6 in prevaccination plasma were significantly elevated in the short-term survivors. 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 inflammation associated with cancer development and progression.[34] There have been many studies describing the correlation between IL-6 levels and prognosis in various types of cancers, including prostate cancer.[56-59] Interestingly, IL-6 has been also shown to rapidly generate myeloid-derived suppressor cells from precursors that are present in murine and human bone marrow or PBMCs, in the presence of other cytokines such as GM-CSF,[60,61] although in the current study, the expression levels of plasma IL-6 were not well correlated with expressions of granulocyte-related genes in the microarray analysis (data not shown). Although the role of IL-6 in the immune responses to cancer vaccines still 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.

To the best of our knowledge, this is the first study to characterize gene expression profiles in peripheral blood and thereby identify biomarkers for predicting clinical outcomes after peptide vaccines. Our findings suggest that the widely available gene expression profiling in peripheral blood may permit future development of molecular-based personalized immunotherapies through discrimination between patients with good and poor prognoses. Although our experimental approaches were not novel, the ability to predict patient prognosis on the basis of relatively simple assays with easily available peripheral blood samples would be of importance. It may be possible that the current study would provide important information for defining eligibility and/or exclusion criteria for personalized peptide vaccination in castration-resistant prostate cancer patients. Nevertheless, because this is a retrospective study with a limited number of patients, all of whom received personalized peptide vaccination, clinical utility of the identified gene signatures and gene classifier needs to be confirmed in future larger-scale, prospective trials conducted in defined patient populations receiving or not receiving personalized peptide vaccination. In addition, the gene expression profiles identified in the current study remain to be verified by using other, independent methods for mRNA and/or protein quantification.

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

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

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

Introduction

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

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

Patients and methods

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

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

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

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

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

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

Flow cytometric analysis of immune subsets among PBMCs. A suppressive immune subset, myeloid-derived suppressor cells (MDSCs), among PBMCs were examined by flow cytometry. For analysis of MDSCs, PBMCs (0.5×10^6) suspended in PBS containing 2% FBS were incubated with the following monoclonal antibodies (Abs) for 30 min at 4°C: anti-CD3-FITC, anti-CD56-FITC, anti-CD19-FITC, anti-CD33-APC, anti-HLA-DR-PE/Cy7, and anti-CD14-APC/Cy7. In the cell subset negative for lineage markers (CD3, CD19, CD56, CD14) and HLA-DR, MDSCs were identified as positive for CD33. The frequency of MDSCs in the lymphocyte gate defined by forward scatter and side scatter was calculated. In addition, the expression of CD26 in PBMCs was also analyzed, since the expression level of this gene assessed by cDNA microarray analysis has been shown to be predictive of OS in patients with prostate cancer receiving PPV (Sasada *et al*, unpublished data).

Table I. Peptide candidates for cancer vaccination.

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

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

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

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

Results

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

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

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

Table II. Continued.

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

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

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

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

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

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

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

Table III. Toxicities.

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

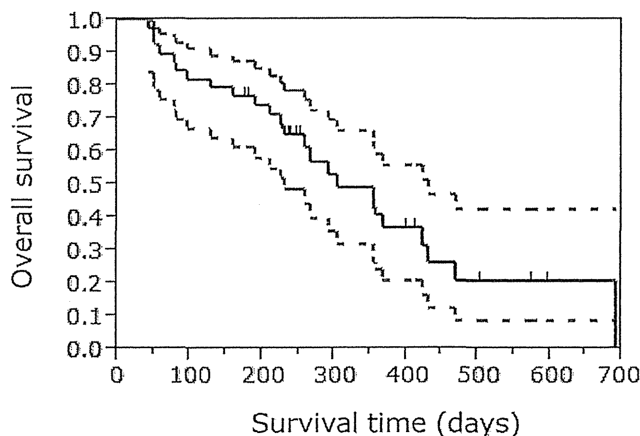

Figure 1. Kaplan-Meier survival analysis in the NSCLC patients receiving PPV. The median overall survival of patients who received PPV (n=41, solid line) was 304 days and the one-year survival rate was 42%. Dotted lines show 95% confidence intervals.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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FOCUSSED RESEARCH REVIEW

Personalized peptide vaccination: a new approach for advanced cancer as therapeutic cancer vaccine

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Abstract Since both tumor cells and host immune cell repertoires are diverse and heterogeneous, immune responses against tumor-associated antigens should differ substantially among individual cancer patients. Selection of suitable peptide vaccines for individual patients based on the preexisting host immunity before vaccination could induce potent anti-tumor responses that provide clinical benefit to cancer patients. We have developed a novel immunotherapeutic approach of personalized peptide vaccination (PPV) in which a maximum of four human leukocyte antigen (HLA) class IA-matched peptides are selected for vaccination among pooled peptides on the basis of both HLA class IA type and the preexisting host immunity before vaccination. In this review, we discuss our recent results of preclinical and clinical studies of PPV for various types of advanced cancer.

Keywords Immunotherapy · Personalized peptide vaccine · Cancer vaccine · Advanced cancer

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Abbreviations

APC	Advanced pancreatic cancer
CR	Complete response
CRPC	Castration-resistant prostate cancer
CTL	Cytotoxic T lymphocytes
DBC	Docetaxel-based chemotherapy
EMP	Estramustine phosphate
GBM	Glioblastoma multiforme
GEM	Gemcitabine
HLA	Human leukocyte antigen
IgG	Immunoglobulin G
MHC	Major histocompatibility complex
MRP3	Multidrug resistance-associated protein 3
MST	Median survival time
NSLC	Non-small cell lung cancer
PBMC	Peripheral blood mononuclear cells
PAP	Prostatic acid phosphatase
PD	Progressive disease
PFS	Progression-free survival
PPV	Personalized peptide vaccine
PR	Partial response
PSA	Prostate-specific antigen
PSMA	Prostate-specific membrane antigen
SART	Squamous cell carcinoma antigen recognized by T cells
SCLC	Small cell lung cancer
TAA	Tumor-associated antigen

Introduction

Since the identification of tumor-associated antigens (TAA) in different tumor histological types, many cancer vaccination strategies have been investigated, including peptide-based vaccines, recombinant DNA- or protein-based

vaccines, and cell-based vaccines. Results from early trials, although demonstrating the feasibility and the good toxicity profile of this approach, provided evidence of clinical activity in only a minority of patients [1]. However, there have recently been noteworthy advances in the clinical application of immunotherapy. In 2010, sipuleucel-T (Provenge; Dendreon Corporation, Seattle, WA, USA), an autologous cellular immunotherapy product designed to stimulate T-cell immune responses against human prostatic acid phosphatase (PAP), was first approved for patients with castration-resistant prostate cancer (CRPC) by the U.S. Food and Drug Administration (FDA) [2]. In addition, another immunotherapeutic agent, ipilimumab, an anti-cytotoxic T-lymphocyte antigen (CTLA)-4 monoclonal antibody, was also approved for melanoma patients by the FDA in 2011 [3]. Despite these significant advances, however, most other randomized clinical trials of immunotherapies, including peptide vaccines, recombinant DNA- or protein-based vaccines, and cell-based vaccines, have so far failed to show beneficial therapeutic effects in patients compared to existing treatments [4]. The failure of recent clinical trials has raised several issues that need to be addressed for the successful development of cancer vaccines. We describe here a novel immunotherapeutic approach, "personalized peptide vaccination (PPV)," in which a maximum of four human leukocyte antigen (HLA) class IA-matched peptides are selected for vaccination from a pool of peptides on the basis of both HLA class IA type and the preexisting host immunity before vaccination. This strategy may confer several advantages, such as the possibility of bypassing both immunological diversity and tumor heterogeneity. For example, "personalized" antigens with preexisting immunity, which are designed to stimulate antigen-specific memory T cells, could be expected to induce rapid and strong secondary immune responses. For example, we previously reported that PPV quickly induced infiltration of $CD45RO^+$ memory T cells, rather than naïve T cells or B cells, into cancer tissues [5]. In addition, selection of multiple epitopes for PPV could reduce the risk of tumor escape through existence and/or induction of antigen-negative clones escaping peptide-specific immune responses. Indeed, it would be relatively rare that tumor cells escape from peptide-specific immune responses by simultaneously losing all of multiple antigens selected for vaccination.

Characteristics of candidate peptides for PPV

A large number of tumor-associated antigens (TAA) have been identified by several different approaches, including complementary DNA (cDNA) expression cloning [6], serologic analysis of recombinant cDNA expression libraries (SEREX) [7], and a reverse immunological approach. We have identified a number of TAA genes and their peptides, some of which have been used as vaccine antigens for PPV, by cDNA expression cloning techniques. For example, a series of the squamous cell carcinoma antigens recognized by T cells (SART), including SART1, SART2, and SART3, were identified from a cDNA library of a squamous cell carcinoma cell line for the first time as TAA derived from epithelial cancers except for melanoma, by using a CTL line established from a patient with esophageal cancer [8–11]. Similarly, other TAAs with interesting characteristics, such as $p56^{lck}$ and multidrug resistance-associated protein 3 (MRP3), have also been identified as vaccine antigen candidates for PPV. $p56^{lck}$, the *src* family tyrosine kinase essential for T-cell development and function is reported to be aberrantly expressed in colon, small cell lung carcinoma, and prostatic cancer cells with a trend toward preferential expression in metastatic cancer cells [12, 13]. This molecule encodes epitopes, which can frequently induce cytotoxic T lymphocytes (CTLs) in the peripheral blood lymphocytes of $HLA\text{-}A2^+$, $HLA\text{-}A24^+$, or HLA-A3 $supertype^+$ cancer patients with distant metastases [14–16]. Peptides derived from MRP3, which are recognized by CTLs in an HLA-A2402-restricted manner [17], often show positive immune responses in advanced cancer patients in whom standard chemotherapy had failed.

One of the notable characteristics of PPV is to screen CTL epitope candidates for therapeutic cancer vaccines on the basis of their ability to induce CTL and/or humoral responses in pre-vaccination samples, since all of the CTL epitopes currently employed for PPV have B-cell epitopes as well. This is based on the hypothesis that a CTL peptide possessing a B-cell epitope could provide more effective clinical benefits than a CTL peptide without it. Although this hypothesis has not been confirmed by randomized clinical trials yet, it has been well recognized that both cellular and humoral immune responses are crucial to induce potent anti-tumor immunity in animal models [18, 19]. As a result of basic and clinical studies, we have focused on 31 HLA class I-restricted peptide epitopes with minimal optimal length for PPV, [2 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for HLA-A3 supertype (A3, A11, A31, or A33), and 4 peptides for HLA-A26] (Table 1). These peptides were identified from 15 different TAAs, including SART2, SART3, $p56^{lck}$, MRP3, prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), prostate-specific membrane antigen (PSMA), and a variety of other epithelial tumor antigens [8–11, 14–17, 20–28]. The safety and potential immunological effects of these vaccine candidates have been shown in previously conducted clinical studies.

Although short peptide epitopes with minimal optimal length have been reported to bear the potential to induce tolerance rather than effective immune responses [29], PPV

Table 1 Information of peptide candidates used for personalized peptide vaccination

Peptide name	Original protein	Position of peptide	Amino acid sequence	HLA type	References
CypB-129	Cyclophilin B	129–138	KLKHYGPGWV	A2, A3sup[a]	[20]
Lck-246	p56 lck	246–254	KLVERLGAA	A2	[15]
Lck-422	p56 lck	422–430	DVWSFGILL	A2, A3sup	[15]
MAP-432	ppMAPkkk	432–440	DLLSHAFFA	A2, A26	[21]
WHSC2-103	WHSC2	103–111	ASLDSDPWV	A2, A3sup[a], A26	[21]
HNRPL-501	HNRPL	501–510	NVLHFFNAPL	A2, A26	[21]
UBE-43	UBE2 V	43–51	RLQEWCSVI	A2	[21]
UBE-85	UBE2 V	85–93	LIADFLSGL	A2	[21]
WHSC2-141	WHSC2	141–149	ILGELREKV	A2	[21]
HNRPL-140	HNRPL	140–148	ALVEFEDVL	A2	[21]
SART3-302	SART3	302–310	LLQAEAPRL	A2	[8]
SART3-309	SART3	309–317	RLAEYQAYI	A2	[8]
SART2-93	SART2	93–101	DYSARWNEI	A24	[9]
SART3-109	SART3	109–118	VYDYNCHVDL	A24, A3sup[a], A26	[10]
Lck-208	p56 lck	208–216	HYTNASDGL	A24	[14]
PAP-213	PAP	213–221	LYCESVHNF	A24	[22]
PSA-248	PSA	248–257	HYRKWIKDTI	A24	[23]
EGFR-800	EGF-R	800–809	DYVREHKDNI	A24	[24]
MRP3-503	MRP3	503–511	LYAWEPSFL	A24	[17]
MRP3-1293	MRP3	1293–1302	NYSVRYRPGL	A24	[17]
SART2-161	SART2	161–169	AYDFLYNYL	A24	[9]
Lck-486	p56 lck	486–494	TFDYLRSVL	A24	[14]
Lck-488	p56 lck	488–497	DYLRSVLEDF	A24	[14]
PSMA-624	PSMA	624–632	TYSVSFDSL	A24	[25]
EZH2-735	EZH2	735–743	KYVGIEREM	A24	[26]
PTHrP-102	PTHrP	102–111	RYLTQETNKV	A24	[27]
SART3-511	SART3	511–519	WLEYYNLER	A3sup[a]	[11]
SART3-734	SART3	734–742	QIRPIFSNR	A3sup[a]	[11]
Lck-90	p56 lck	90–99	ILEQSGEWWK	A3sup[a]	[16]
Lck-449	p56 lck	449–458	VIQNLERGYR	A3sup[a]	[16]
PAP-248	PAP	248–257	GIHKQKEKSR	A3sup[a]	[28]

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

using short epitopes has been reported to efficiently induce antigen-specific IFN-γ-producing $CD8^+$ T cells with cytotoxic activity, but not tolerance to them, possibly because only immunogenic epitopes are selected in each patient by pre-vaccination screening. Although long peptides have shown excellent immune and clinical responses in some of clinical trials [30], we do not currently employ long peptides for PPV, since it may be possible that they contain undesirable T-cell epitopes that stimulate immune suppressive cells, such as regulatory T cells or T helper-2 cells [31], which may negatively regulate beneficial immune and clinical responses.

For PPV, a maximum of 4 peptides, selected on the basis of the results of HLA typing and the preexisting immune responses specific to each of the 31 different vaccine candidates, are subcutaneously administered in complex with incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France) weekly or biweekly. To prevent interaction/competition among peptides at the vaccinated sites, each of vaccine peptides is injected separately at different sites, but not in a mixture at a single site. Since more than five peptides per vaccination seemed intolerable due to adverse skin reactions, which sometimes may cause unpleasant symptoms, such as itching and pain, in our previous feasibility studies (unpublished data), four peptides per vaccination have currently been employed. Regarding the vaccination schedule, selected peptides are administered at the weekly schedule at least for the first cycle of six vaccinations, since a clear trend toward better immune responses was observed among the patients who underwent the weekly administration protocol, compared to the biweekly protocol in previous clinical trials [32].

Rationale for PPV

Although the number of cancer vaccine candidates is becoming almost limitless, antigen peptides employed for vaccination against individual patients might not always be appropriate. In general, anti-tumor immunity is known to be dependent on both the immunological characteristics of tumor cells and the host immune cell repertoires. Since immune cell repertoires of the hosts are quite diverse and heterogeneous, anti-tumor immunity might differ substantially among individuals. Therefore, it is likely that vaccine antigens that are selected and administered without considering the host immune cell repertoires would not efficiently induce beneficial anti-tumor immune responses. To increase the clinical benefits from cancer vaccines, particular attention should be paid to the immunological status of each patient by characterizing the preexisting immune responses to vaccine antigens before vaccination. However, in most of the current clinical trials of therapeutic cancer vaccines, common antigens are employed for vaccination independently of the immunological status of patients.

Patients who have an immunological memory to vaccine antigens are expected to show quick and strong immune responses to them. In contrast, patients with no immunological memory against vaccine antigens would take more time to develop effective anti-tumor immune responses because several rounds of repeated vaccinations might be required to prime antigen-specific naive T cells to functional effector cells (Fig. 1). In such situations, vaccinations could not easily provide clinical benefits, especially in advanced cancer patients who show a relatively quick disease progression. Moreover, immune responses induced by inadequate vaccines that are non-specific to tumor cells may not only be ineffective for tumor control, but also may erode preexisting immunity. On the basis of the current paradigm that the size and composition of the adaptive immune system are limited and that individual immune cells are constantly competing with each other in a limited space, inadequate vaccination may have negative consequences for the host by suppressing preexisting beneficial memory cells specific to tumors and/or infections, which might result in acceleration of cancer progression or early death in vaccinated patients. In addition, the approach, which is designed to stimulate antigen-specific memory T cells, but not to prime naïve T cells, might not need additional immune boosting, such as the blocker of checkpoint molecule, CTLA-4, since it has been known that memory T cells are less dependent on costimulatory molecules for recall responses [33, 34]. Considering these issues, it would be quite reasonable for vaccine antigens to be selected on the basis of the preexisting immunological status in each patient.

In addition, it should be noted that cancer cells possess or develop a variety of mechanisms to maintain their malignant behavior. For example, it has been well recognized that cancer cells escape from host immunological surveillance [35]. Through the interaction between the host immune system and tumor cells at the equilibrium phase, immunological pressure often produces tumor cell variants that decrease or lose tumor-associated antigens. Therefore, for better control of cancer cells, it would be recommended to administer multiple tumor-associated antigens to reduce the risk of outgrowth of antigen-loss variants.

In view of the complexity and diversity of the immune cell repertoires of hosts and the immunological characteristics of tumors, we have developed the new concept of

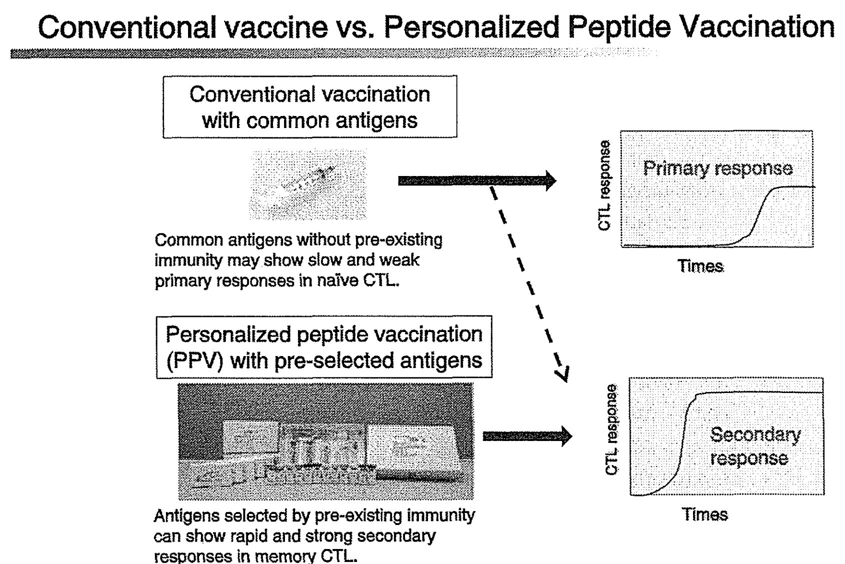

Fig. 1 Concept of personalized peptide vaccination. Patients who have an immunological memory to vaccine antigens are expected to show quick and strong immune responses to them. In contrast, patients with no immunological memory against vaccine antigens would take more time to develop effective anti-tumor immune responses because several rounds of repeated vaccinations might be required to prime antigen-specific naïve T cells to functional effector cells

PPV. In this "personalized" cancer vaccine formulation, multiple peptide antigens appropriate for vaccination are screened and selected from a list of pooled vaccine candidates in each patient, based on preexisting host immunity. In the early-phase translational study of PPV, the preexisting immunity was defined by the frequencies of CTL precursors in pre-vaccination PBMCs by using the peptide-specific IFN-γ production assay with the cutoff level of around 1 of 10,000 cells as reported, since we found that the magnitude of CTL activation could be in part dependent on the frequencies of peptide-specific CTL precursors in circulation, which were determined by this assay [36]. Indeed, when CTL precursors were measured in pre-vaccination PBMC followed by administration of peptides with higher CTL precursor frequencies, rapid and strong activation of CTL with potential clinical benefits was induced in certain patients of a series of clinical trials for advanced cancers [32, 37, 38].

Nevertheless, we are currently evaluating the preexisting immunity to vaccine candidates by peptide-specific immunoglobulin G (IgG) responses in pre-vaccination plasma, which are determined by the multiplex bead-based LUMINEX assay with the cutoff level of 10 FIU [39], rather than by CTL responses, since we have found that the IgG-based selection is useful for predicting CTL boosting after vaccination in our clinical trials, which showed the safety, high immunogenicity, and possible clinical benefits of PPV. For example, in the phase I trial of PPV for recurrent or progressive glioblastoma patients, CTL responses were boosted in 23 of 48 vaccinated peptides (48 %), which were chosen solely by humoral responses [40]. In addition, CTL responses were induced in 16 of 28 vaccinated peptides (57 %), which were chosen solely by humoral responses in another phase I trial of PPV for castration-resistant prostate cancer (CRPC) patients [41]. Based on these results, the prediction power of evaluating the preexisting immunity solely by the humoral responses for the existence of CTL responses could be estimated around 50 % when four peptides were chosen for the vaccination. Figure 2 shows one example of original data for determining the preexisting immune responses before vaccination by the humoral responses to vaccine candidates. As shown in this figure, immune responses to multiple peptides can be detected before vaccination in most of the patients treated with PPV. In such situation, the peptides showing higher IgG responses are selected for vaccination from the list of peptides that match the patient's HLA types.

There are some reasons for assessing IgG responses, instead of CTL responses, to define the preexisting immune responses. The most critical reason is a technical issue that standard methods to measure the CTL activity have not been well established yet. The performance characteristics, such as sensitivity and reproducibility, of currently available CTL assays require more modification/sophistication to detect low frequencies of antigen-specific CTL, and it seems difficult to validate the quality of the assays in clinical trials [42, 43]. In contrast, the multiplex bead-based

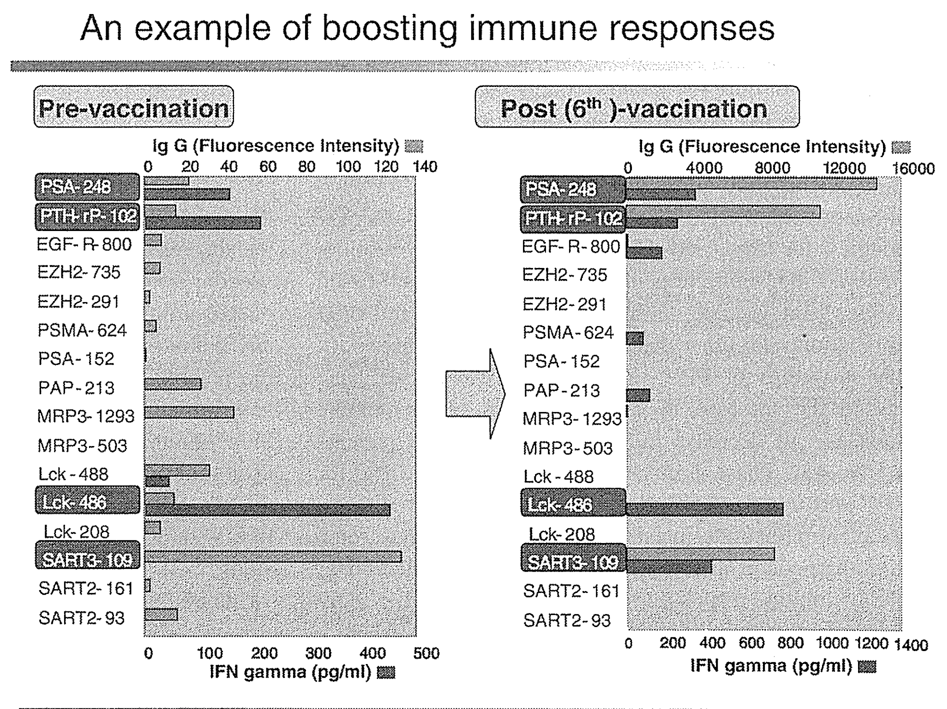

Fig. 2 An example of boosting immune responses. IgG responses to PSA-248 peptide increased from 20 to 14,000 FIU in the post (6th)-vaccination samples. The similar boosting was observed in the other two peptides. CTL response was also increased in all four vaccinated peptides. Clinical response of this case was PR