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

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).

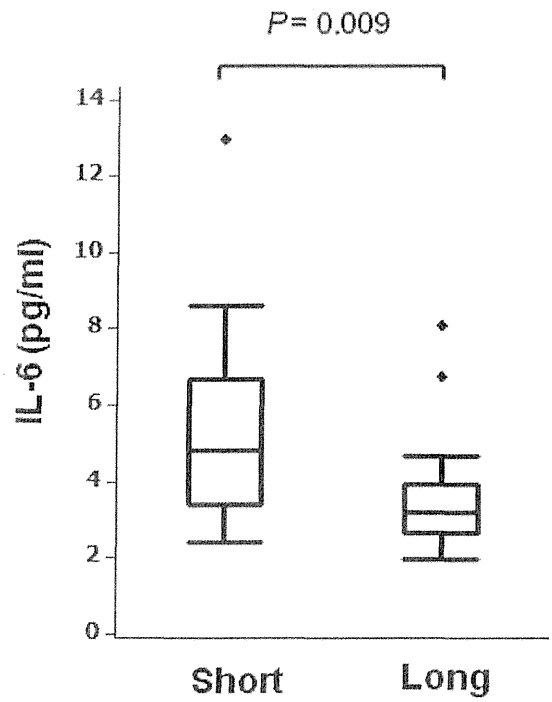

Figure 3. Increase in plasma interleukin (IL)-6 levels in the short-term survivors is shown. The levels of IL-6 assessed by bead-based multiplex assay in prevaccination plasma were compared between the short-term (n = 18) and long-term (n = 18) survivors. Box plots show median and interquartile range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

FUNDING SOURCES

This study was supported by the grant, Regional Innovation Cluster Program of the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to K.I.).

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

REFERENCES

1. Finn OJ. Cancer immunology. *N Engl J Med.* 2008;358:2704-2715.
2. Schwartzentruber DJ, Lawson DH, Richards JM, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N Engl J Med.* 2011;364:2119-2127.
3. Kantoff PW, Higano CS, Shore ND, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med.* 2010;363:411-422.
4. Kenter GG, Welters MJ, Valentijn AR, et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med.* 2009;361:1838-1847.
5. Sasada T, Komatsu N, Suekane S, Yamada A, Noguchi M, Itoh K. Overcoming the hurdles of randomised clinical trials of therapeutic cancer vaccines. *Eur J Cancer.* 2010;46:1514-1519.
6. Butterfield LH, Palucka AK, Britten CM, et al. Recommendations from the iSBTc-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers. *Clin Cancer Res.* 2011;17:3064-3076.
7. Hoos A, Eggermont AM, Janetzki S, et al. Improved endpoints for cancer immunotherapy trials. *J Natl Cancer Inst.* 2010;102:1388-1397.
8. Disis ML. Immunologic biomarkers as correlates of clinical response to cancer immunotherapy. *Cancer Immunol Immunother.* 2011;60:433-442.
9. Ugurel S, Schrama D, Keller G, et al. Impact of the CCR5 gene polymorphism on the survival of metastatic melanoma patients receiving immunotherapy. *Cancer Immunol Immunother.* 2008;57:685-691.
10. Liu D, O'Day SJ, Yang D, et al. Impact of gene polymorphisms on clinical outcome for stage IV melanoma patients treated with biochemotherapy: an exploratory study. *Clin Cancer Res.* 2005;11:1237-1246.
11. Leibovici D, Grossman HB, Dinney CP, et al. Polymorphisms in inflammation genes and bladder cancer: from initiation to recurrence, progression, and survival. *J Clin Oncol.* 2005;23:5746-5756.
12. Breunis WB, Tarazona-Santos E, Chen R, Kiley M, Rosenberg SA, Chanock SJ. Influence of cytotoxic T lymphocyte-associated antigen 4 (CTLA4) common polymorphisms on outcome in treatment of melanoma patients with CTLA-4 blockade. *J Immunother.* 2008;31:586-590.

13. Yurkovetsky ZR, Kirkwood JM, Edington HD, et al. Multiplex analysis of serum cytokines in melanoma patients treated with interferon-alpha2b. *Clin Cancer Res.* 2007;13:2422-2428.
14. Sabatino M, Kim-Schulze S, Panelli MC, et al. Serum vascular endothelial growth factor and fibronectin predict clinical response to high-dose interleukin-2 therapy. *J Clin Oncol.* 2009;27:2645-2652.
15. van de Vijver MJ, He YD, van't Veer LJ, et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med.* 2002;347:1999-2009.
16. Bedognetti D, Wang E, Sertoli MR, Marincola FM. Gene-expression profiling in vaccine therapy and immunotherapy for cancer. *Expert Rev Vaccines.* 2010;9:555-565.
17. Bogunovic D, O'Neill DW, Belitskaya-Levy I, et al. Immune profile and mitotic index of metastatic melanoma lesions enhance clinical staging in predicting patient survival. *Proc Natl Acad Sci U S A.* 2009;106:20429-20434.
18. Pham MX, Teuteberg JJ, Kfoury AG, et al. Gene-expression profiling for rejection surveillance after cardiac transplantation. *N Engl J Med.* 2010;362:1890-1900.
19. Chaussabel D, Pascual V, Banchereau J. Assessing the human immune system through blood transcriptomics. *BMC Biol.* 2010;8:84.
20. Newell KA, Asare A, Kirk AD, et al. Identification of a B cell signature associated with renal transplant tolerance in humans. *J Clin Invest.* 2010;120:1836-1847.
21. Itoh K, Yamada A. Personalized peptide vaccines: a new therapeutic modality for cancer. *Cancer Sci.* 2006;97:970-976.
22. Noguchi M, Kakuma T, Uemura H, et al. A randomized phase II trial of personalized peptide vaccine plus low dose estramustine phosphate (EMP) versus standard dose EMP in patients with castration resistant prostate cancer. *Cancer Immunol Immunother.* 2010;59:1001-1009.
23. Terasaki M, Shibui S, Narita Y, et al. Phase I trial of a personalized peptide vaccine for patients positive for human leukocyte antigen-A24 with recurrent or progressive glioblastoma multiforme. *J Clin Oncol.* 2011;29:337-344.
24. Noguchi M, Mine T, Komatsu N, et al. Assessment of immunological biomarkers in patients with advanced cancer treated by personalized peptide vaccination. *Cancer Biol Ther.* 2011;10:1266-1279.
25. Higano CS, Schellhammer PF, Small EJ, et al. Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with sipuleucel-T in advanced prostate cancer. *Cancer.* 2009;115:3670-3679.
26. Tannock IF, de Wit R, Berry WR, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med.* 2004;351:1502-1512.
27. Petrylak DP, Tangen CM, Hussain MH, et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med.* 2004;351:1513-1520.
28. Berthold DR, Pond GR, Soban F, de Wit R, Eisenberger M, Tannock IF. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer: updated survival in the TAX 327 study. *J Clin Oncol.* 2008;26:242-245.
29. Shi L, Reid LH, Jones WD, et al. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat Biotechnol.* 2006;24:1151-1161.
30. Rodriguez PC, Ochoa AC. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. *Immunol Rev.* 2008;222:180-191.
31. Tartour E, Pere H, Maillere B, et al. Angiogenesis and immunity: a bidirectional link potentially relevant for the monitoring of antiangiogenic therapy and the development of novel therapeutic combination with immunotherapy. *Cancer Metastasis Rev.* 2011;30:83-95.
32. Bissell MJ, Hines WC. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nat Med.* 2011;17:320-329.
33. Disis ML. Immune regulation of cancer. *J Clin Oncol.* 2010;28:4531-4538.
34. Naugler WE, Karin M. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. *Trends Mol Med.* 2008;14:109-119.
35. Davis JM III, Knutson KL, Strausbauch MA, et al. Analysis of complex biomarkers for human immune-mediated disorders based on cytokine responsiveness of peripheral blood cells. *J Immunol.* 2010;184:7297-7304.
36. Sousa I, Clark TG, Holt R, et al. Polymorphisms in leucine-rich repeat genes are associated with autism spectrum disorder susceptibility in populations of European ancestry. *Mol Autism.* 2010;1:7.
37. Haruki S, Imoto I, Kozaki K, et al. Frequent silencing of protocadherin 17, a candidate tumour suppressor for esophageal squamous cell carcinoma. *Carcinogenesis.* 2010;31:1027-1036.
38. Balakrishnan L, Milavetz B. Decoding the histone H4 lysine 20 methylation mark. *Crit Rev Biochem Mol Biol.* 2010;45:440-452.
39. Dziarski R, Gupta D. Review: Mammalian peptidoglycan recognition proteins (PGRPs) in innate immunity. *Innate Immun.* 2010;16:168-174.
40. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res.* 2001;61:4756-4760.
41. Rodriguez PC, Ernstoff MS, Hernandez C, et al. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer Res.* 2009;69:1553-1560.
42. Brandau S, Trellakis S, Bruderek K, et al. Myeloid-derived suppressor cells in the peripheral blood of cancer patients contain a subset of immature neutrophils with impaired migratory properties. *J Leukoc Biol.* 2010;89:311-317.
43. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol.* 2009;9:162-174.
44. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol.* 2009;182:4499-4506.
45. Peranzoni E, Zilio S, Marigo I, et al. Myeloid-derived suppressor cell heterogeneity and subset definition. *Curr Opin Immunol.* 2010;22:238-244.
46. Gregory AD, Houghton AM. Tumor-associated neutrophils: new targets for cancer therapy. *Cancer Res.* 2011;71:2411-2416.
47. Fridlender ZG, Sun J, Kim S, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell.* 2009;16:183-194.
48. Houghton AM, Rzymkiewicz DM, Ji H, et al. Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. *Nat Med.* 2010;16:219-223.
49. Jablonska J, Leschner S, Westphal K, Lienenklaus S, Weiss S. Neutrophils responsive to endogenous IFN-beta regulate

tumor angiogenesis and growth in a mouse tumor model. *J Clin Invest.* 2010;120:1151-1164.
50. Wislez M, Rabbe N, Marchal J, et al. Hepatocyte growth factor production by neutrophils infiltrating bronchioloalveolar subtype pulmonary adenocarcinoma: role in tumor progression and death. *Cancer Res.* 2003;63:1405-1412.
51. Jensen HK, Donskov F, Marcussen N, Nordsmark M, Lundbeck F, von der Maase H. Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. *J Clin Oncol.* 2009;27:4709-4717.
52. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res.* 2005;11:6713-6721.
53. Vincent J, Mignot G, Chalmin F, et al. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer Res.* 2010;70:3052-3061.
54. Ko JS, Zea AH, Rini BI, et al. Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. *Clin Cancer Res.* 2009;15:2148-2157.
55. Lebbink RJ, van den Berg MC, de Ruiter T, et al. The soluble leukocyte-associated Ig-like receptor (LAIR)-2 antagonizes the collagen/LAIR-1 inhibitory immune interaction. *J Immunol.* 2008;180:1662-1669.
56. Scambia G, Testa U, Benedetti Panici P, et al. Prognostic significance of interleukin 6 serum levels in patients with ovarian cancer. *Br J Cancer.* 1995;71:354-356.
57. Nakashima J, Tachibana M, Horiguchi Y, et al. Serum interleukin 6 as a prognostic factor in patients with prostate cancer. *Clin Cancer Res.* 2000;6:2702-2706.
58. Okada S, Okusaka T, Ishii H, et al. Elevated serum interleukin-6 levels in patients with pancreatic cancer. *Jpn J Clin Oncol.* 1998;28:12-15.
59. Duffy SA, Taylor JM, Terrell JE, et al. Interleukin-6 predicts recurrence and survival among head and neck cancer patients. *Cancer.* 2008;113:750-757.
60. Marigo I, Bosio E, Solito S, et al. Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. *Immunity.* 2010;32:790-802.
61. Lechner MG, Liebertz DJ, Epstein AL. Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. *J Immunol.* 2010;185:2273-2284.

Personalized peptide vaccination for advanced biliary tract cancer: IL-6, nutritional status and pre-existing antigen-specific immunity as possible biomarkers for patient prognosis

MUNEHIRO YOSHITOMI[1], SHIGERU YUTANI[2], SATOKO MATSUEDA[2], TETSUYA IOJI[2], NOBUKAZU KOMATSU[2], SHIGEKI SHICHIJO[2], AKIRA YAMADA[3], KYOGO ITOH[2], TETSURO SASADA[2] and HISAFUMI KINOSHITA[1]

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

Received August 29, 2011; Accepted November 22, 2011

DOI: 10.3892/etm.2011.424

Abstract. Considering that the prognosis of patients with advanced biliary tract cancer (BTC) remains very poor, with a median survival of less than 1 year, new therapeutic approaches need to be developed. In the present study, a phase II clinical trial of personalized peptide vaccination (PPV) was conducted in advanced BTC patients to evaluate the feasibility of this treatment and to identify potential biomarkers. A maximum of 4 human leukocyte antigen-matched peptides, which were selected based on the pre-existing host immunity prior to vaccination, were subcutaneously administered (weekly for 6 consecutive weeks and bi-weekly thereafter) to 25 advanced BTC patients without severe adverse events. Humoral and/or T cell responses specific to the vaccine antigens were substantially induced in a subset of the vaccinated patients. As shown by multivariate Cox regression analysis, lower interleukin-6 (IL-6) and higher albumin levels prior to vaccination and greater numbers of selected vaccine peptides were significantly favorable factors for overall survival [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]. Based on the safety profile and substantial immune responses to vaccine antigens, PPV could be a promising approach for refractory BTC, although its clinical efficacy remains to be investigated in larger-scale prospective studies. The identified biomarkers are potentially useful for selecting BTC patients who would benefit from PPV.

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

Key words: peptide vaccine, biliary tract cancer, biomarker

Introduction

Biliary tract cancer (BTC) is one of the most aggressive types of cancer and has a very poor prognosis (1,2). Only 10% of newly diagnosed patients present with early-stage disease, which may be treated by a potentially radical excision of the tumor, and the remaining patients have unresectable disease with locally advanced and/or metastatic tumors. Recently, there have been substantial advances in treatment modalities, including systemic chemotherapies, for advanced BTC (1-4). For example, a randomized trial has suggested that cisplatin plus gemcitabine could be considered as a standard treatment option for patients with advanced BTC (3). In addition, a number of different targeted therapies for BTC have also been under investigation (1-4). Despite this progress, however, the prognosis of BTC patients remains very poor, with a median survival of less than 1 year. Therefore, further novel therapeutic approaches need to be developed.

We previously devised a new regime of peptide-based vaccination, known as 'personalized peptide vaccination (PPV)', in which vaccine antigens are selected and administered based on the pre-existing host immunity prior to vaccination (5-7). We reported favorable clinical and/or immune responses of this novel vaccination in various types of advanced cancer, including pancreatic, gastric, colorectal and prostate cancer, and glioblastoma (8-12). For example, a recently conducted randomized clinical trial of PPV for advanced prostate cancer patients showed a promising clinical outcome in the vaccinated group (11). In the present study, we addressed the feasibility of using PPV in advanced BTC patients in a small-scale phase II study. In addition, we identified potential biomarkers for predicting overall survival (OS) and selecting suitable patients for this treatment.

Patients and methods

Patients. Patients were eligible for inclusion in the present study if they had a histological diagnosis of BTC and showed positive humoral responses to at least two of the 31 different vaccine candidate peptides (Table I). Other inclusion criteria

Table I. Peptide candidates for cancer vaccination.

Symbol for peptide	Origin protein	Position of peptide	Amino acid sequence	HLA type
CypB-129	Cyclophilin B	129-138	KLKHYGPGWV	A2, A3sup[a]
Lck-246	p56 lck	246-254	KLVERLGAA	A2
Lck-422	p56 lck	422-430	DVWSFGILL	A2, A3sup
MAP-432	ppMAPkkk	432-440	DLLSHAFFA	A2, A26
WHSC2-103	WHSC2	103-111	ASLDSDPWV	A2, A3sup[a], 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[a], A26
Lck-208	p56 lck	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	p56 lck	486-494	TFDYLRSVL	A24
Lck-488	p56 lck	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[a]
SART3-734	SART3	734-742	QIRPIFSNR	A3sup[a]
Lck-90	p56 lck	90-99	ILEQSGEWWK	A3sup[a]
Lck-449	p56 lck	449-458	VIQNLERGYR	A3sup[a]
PAP-248	PAP	248-257	GIHKQKEKSR	A3sup[a]

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

were as follows: age between 20 and 80 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; positive status for human leukocyte antigen (HLA)-A2, -A24, -A3 supertype (A3, A11, A31 or A33), or -A26; life expectancy of at least 12 weeks; negative status for hepatitis B and C virus; and adequate hematological, hepatic and renal function. Exclusion criteria included pulmonary, cardiac or other systemic diseases; an acute infection; a history of severe allergic reactions; pregnancy or nursing; and 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 2907). Following a full explanation of the protocol, written informed consent was obtained from all patients prior to enrollment.

Clinical protocol. This was an open-label phase II study, in which the primary and secondary end-points were to identify biomarkers for OS and to evaluate the safety of PPV in BTC patients, respectively. In this study, 31 peptides, whose safety and immunological effects had been confirmed in previously conducted clinical studies (6-12), were employed for vaccination [12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for the HLA-A3 supertype (A3, A11, A31 or A33) and 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, USA) and the American Peptide Company (Vista, CA, USA). The right peptides for vaccination to individual patients were selected, taking into consideration the pre-existing host immunity prior to vaccination, assessed by titers of IgG specific to each of the 31 different vaccine candidates, as reported previously (6-12). A maximum of 4 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 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 at 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 Ver. 3.0). Complete blood counts and serum biochemistry tests were performed after every 6 vaccinations. The clinical responses were evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST) in the vaccinated patients, whose radiological findings by computed tomography (CT) scan or magnetic resonance imaging (MRI) were available prior to and following vaccinations.

Measurement of humoral and T cell responses specific to the vaccine peptides. The humoral responses specific to the vaccine peptides were determined by peptide-specific IgG titers using a bead-based multiplex assay with the Luminex 200 system (Luminex, Austin, TX, USA), as reported previously (13). If peptide-specific IgG titers to at least one of the vaccine peptides in the post-vaccination plasma were more than 2-fold 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) using peripheral blood mononuclear cells (PBMCs). Briefly, PBMCs ($2.5x10^4$ cells/well) were incubated in 384-well microculture plates (Iwaki, Tokyo, Japan) with 25 μl of medium (OpTmizer™ T Cell Expansion SFM; Invitrogen, Carlsbad, CA, USA) containing 10% FBS (MP Biologicals, Solon, OH, USA), recombinant human interleukin (IL)-2 (20 IU/ml; Serotec, Oxford, UK) and 10 μM of each peptide. Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) after 3 days of culture. 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 a negative control peptide from human immunodeficiency virus (HIV). Antigen-specific IFN-γ secretion after an 18-h incubation was determined by ELISPOT assay with the Zeiss ELISPOT reader (Carl Zeiss MicroImaging Japan, Tokyo, Japan). Antigen-specific T cell responses were evaluated by the difference between the spot numbers (mean of duplicate samples) in response to the corresponding peptides and those in response to the control peptide. The differences of at least 10 spot numbers per 10^5 PBMCs were considered significant. If the spot numbers in response to at least one of the vaccine peptides in the post-vaccination PBMCs were more than 2-fold higher than those in the pre-vaccination PBMCs, the changes were considered significant.

Measurement of C-reactive protein (CRP), serum amyloid A (SAA) and cytokines. The levels of CRP, SAA and IL-6 in the plasma were examined by ELISA using kits from R&D Systems (Minneapolis, MN, USA), Invitrogen and eBioscience (San Diego, CA, USA), respectively. Bead-based multiplex assays were used to measure Th1/Th2 cytokines, including IL-2, IL-4, IL-5 and IFN-γ (Invitrogen) with the Luminex 200 system. Frozen plasma samples were thawed, diluted and assayed in duplicate in accordance with the manufacturer's instructions. The mean of duplicate samples was used for statistical analysis.

Flow cytometric analysis of suppressive immune subsets in PBMCs. Suppressive immune subsets, myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Treg) in PBMCs were examined by flow cytometry. For analysis of MDSCs, PBMCs ($0.5x10^6$ cells) were stained with the following monoclonal antibodies 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 (all from Biolegend, San Diego, CA, USA). In the cell subpopulation negative for the lineage markers (CD3, CD19, CD56 and CD14) 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. For analysis of Treg, PBMCs ($1x10^6$ cells) were stained with the cocktail of anti-CD4-FITC and anti-CD25-APC, and subsequently with anti-Foxp3-PE following fixation and permeabilization, according to the manufacturer's instructions (eBioscience). The frequency of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+$ cells was calculated. The samples were run on a FACSCanto II (BD Biosciences, San Diego, CA, USA), and data were analyzed using the Diva software (BD Biosciences).

Statistical methods. The two-sided Wilcoxon test was used to compare differences between pre- and post-vaccination measurements. OS time was calculated from the first day of peptide vaccination until the date of mortality or the last date when the patient was known to be alive. Predictive factors for OS were evaluated by univariate and multivariate analyses with the Cox proportional hazards regression model. P-values <0.05 were considered to indicate a statistically significant difference. All the statistical analyses were conducted using the SAS version 9.1 software (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics. Between November 2008 and December 2010, 25 BTC patients were enrolled in the present study. Table II shows the clinicopathological characteristics of the enrolled patients. There were 18 male and 7 female subjects, with a median age of 59 years, ranging from 37 to 79 years. Primary sites of BTC were 7 gallbladder carcinomas, 11 extrahepatic and 6 intrahepatic cholangiocarcinomas, and 1 periampullary carcinoma. All the patients had advanced-stage cancer (stage IVa, n=5; stage IVb, n=9; recurrent, n=11). Prior to enrollment, 22 patients failed to respond to 1 (n=13) or 2 (n=9) regimen(s) of chemotherapy, whereas the remaining 3 patients did not tolerate chemotherapy due to adverse events. The median duration of chemotherapy prior to the PPV was 4 months, ranging from 2 to 27 months. The performance status at the time of enrollment was grade 0 (n=20) or grade 1 (n=5). The numbers of peptides vaccinated to the patients at the first cycle of vaccination were 4 peptides in 19 patients, 3 in 5 patients and 2 in 1 patient. The median number of vaccinations was 10, with a range of 2 to 24. During

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 cholangiocarcionma; 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.

Acknowledgements

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

References

1. Yachimski P and Pratt DS: Cholangiocarcinoma: natural history, treatment, and strategies for surveillance in high-risk patients. J Clin Gastroenterol 42: 178-190, 2008.
2. Hezel AF, Deshpande V and Zhu AX: Genetics of biliary tract cancers and emerging targeted therapies. J Clin Oncol 28: 3531-3540, 2010.
3. Valle J, Wasan H, Palmer DH, *et al*: Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. N Engl J Med 362: 1273-1281, 2010.
4. Gruenberger B, Schueller J, Heubrandtner U, *et al*: Cetuximab, gemcitabine, and oxaliplatin in patients with unresectable advanced or metastatic biliary tract cancer: a phase 2 study. Lancet Oncol 11: 1142-1148, 2010.
5. Sasada T, Komatsu N, Suekane S, Yamada A, Noguchi M and Itoh K: Overcoming the hurdles of randomised clinical trials of therapeutic cancer vaccines. Eur J Cancer 46: 1514-1519, 2010.
6. Mine T, Sato Y, Noguchi M, *et al*: Humoral responses to peptides correlate with overall survival in advanced cancer patients vaccinated with peptides based on pre-existing, peptide-specific cellular responses. Clin Cancer Res 10: 929-937, 2004.
7. Itoh K and Yamada A: Personalized peptide vaccines: a new therapeutic modality for cancer. Cancer Sci 97: 970-976, 2006.
8. Yanagimoto H, Shiomi H, Satoi S, *et al*: A phase II study of personalized peptide vaccination combined with gemcitabine for non-resectable pancreatic cancer patients. Oncol Rep 24: 795-801, 2010.
9. Sato Y, Fujiwara T, Mine T, *et al*: Immunological evaluation of personalized peptide vaccination in combination with a 5-fluorouracil derivative (TS-1) for advanced gastric or colorectal carcinoma patients. Cancer Sci 98: 1113-1119, 2007.
10. Hattori T, Mine T, Komatsu N, *et al*: Immunological evaluation of personalized peptide vaccination in combination with UFT and UZEL for metastatic colorectal carcinoma patients. Cancer Immunol Immunother 58: 1843-1852, 2009.
11. Noguchi M, Kakuma T, Uemura H, *et al*: A randomized phase II trial of personalized peptide vaccine plus low dose estramustine phosphate (EMP) versus standard dose EMP in patients with castration resistant prostate cancer. Cancer Immunol Immunother 59: 1001-1009, 2010.
12. Terasaki M, Shibui S, Narita Y, *et al*: Phase I trial of a personalized peptide vaccine for patients positive for human leukocyte antigen – A24 with recurrent or progressive glioblastoma multiforme. J Clin Oncol 29: 337-344, 2011.
13. Komatsu N, Shichijo S, Nakagawa M and Itoh K: New multiplexed flow cytometric assay to measure anti-peptide antibody: a novel tool for monitoring immune responses to peptides used for immunization. Scand J Clin Lab Invest 64: 535-545, 2004.
14. Noguchi M, Mine T, Komatsu N, *et al*: Assessment of immunological biomarkers in patients with advanced cancer treated by personalized peptide vaccination. Cancer Biol Ther 10: 1266-1279, 2011.
15. Disis ML: Immunologic biomarkers as correlates of clinical response to cancer immunotherapy. Cancer Immunol Immunother 60: 433-442, 2011.
16. Butterfield LH, Palucka AK, Britten CM, *et al*: Recommendations from the iSBTc-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers. Clin Cancer Res 17: 3064-3076, 2011.
17. Hoos A, Eggermont AM, Janetzki S, *et al*: Improved endpoints for cancer immunotherapy trials. J Natl Cancer Inst 102: 1388-1397, 2010.
18. Naugler WE and Karin M: The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends Mol Med 14: 109-119, 2008.
19. Scambia G, Testa U, Benedetti Panici P, *et al*: Prognostic significance of interleukin 6 serum levels in patients with ovarian cancer. Br J Cancer 71: 354-356, 1995.
20. Nakashima J, Tachibana M, Horiguchi Y, *et al*: Serum interleukin 6 as a prognostic factor in patients with prostate cancer. Clin Cancer Res 6: 2702-2706, 2000.
21. Okada S, Okusaka T, Ishii H, *et al*: Elevated serum interleukin-6 levels in patients with pancreatic cancer. Jpn J Clin Oncol 28: 12-15, 1998.
22. Goydos JS, Brumfield AM, Frezza E, Booth A, Lotze MT and Carty SE: Marked elevation of serum interleukin-6 in patients with cholangiocarcinoma: validation of utility as a clinical marker. Ann Surg 227: 398-404, 1998.
23. Marigo I, Bosio E, Solito S, *et al*: Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. Immunity 32: 790-802, 2010.
24. Lechner MG, Liebertz DJ and Epstein AL: Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. J Immunol 185: 2273-2284, 2010.
25. Zou W and Restifo NP: T(H)17 cells in tumour immunity and immunotherapy. Nat Rev Immunol 10: 248-256, 2010.

Cancer Science
The official journal of the Japanese Cancer Association JCA

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

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

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

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

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

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

Materials and Methods

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

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

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

doi: 10.1111/j.1349-7006.2012.02202.x

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

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

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

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

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

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

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

Results

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

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

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

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

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

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

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

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

doi: 10.1111/j.1349-7006.2012.02202.x

Table 2. Toxicities

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

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

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

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

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

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

Table 3. Immunological responses to the vaccine peptides

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

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

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

Table 4. Laboratory data before and after vaccination†

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

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

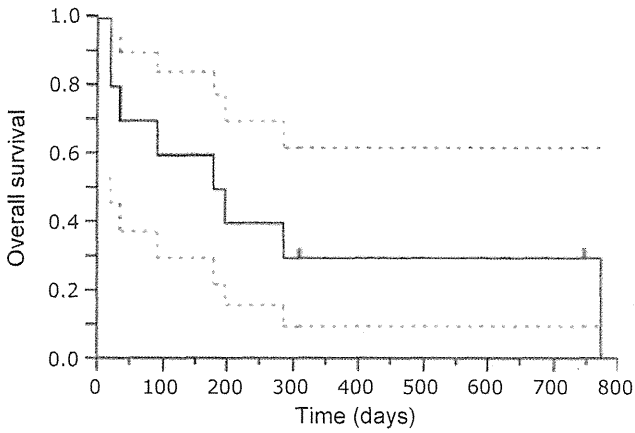

Fig. 1. Kaplan–Meier survival analysis in the enrolled patients. The median overall survival of patients who received personalized peptide vaccination (PPV) (n = 10; solid line) was 186.5 days and the 1 year survival rate was 30%. Dotted lines show 95% confidence intervals.

Table 5. Statistical analysis with clinical findings and laboratory data

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

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

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

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

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

Discussion

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

doi: 10.1111/j.1349-7006.2012.02202.x