more than twofold higher than those in the pre-vaccination plasma, the changes were considered to be significant. If a significant increase was observed in at least one vaccinated peptide, the specific humoral response was considered to be augmented.

Although T-cell subsets using flowcytometry was not analyzed in this study, T-cell responses specific to the vaccinated peptides were evaluated by INF-γ ELISPOT using peripheral blood mononuclear cells (PBMCs), which were separated from peripheral blood (30 ml) by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare; Uppsala, Sweden) and stored frozen until analysis. After thawing, PBMCs (2.5×10^4 cells/well) were incubated in 384-well microculture plates (IWAKI, Tokyo, Japan) with 25 μl of medium (OpTmizer™ T Cell Expansion SFM; Invitrogen, Carlsbad, CA) containing 10% FBC (MP Biologicals, Solon, OH), IL-2 (20 IU/ml; Serotec, Oxford, UK), and each peptide (10 μM). Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) after culturing for 3 days. After incubating for the following 6 days, the cells were harvested and tested for their ability to produce interferon (IFN)-γ in response to either the corresponding peptides or a negative control peptide human immunodeficiency virus (sequences: SLYNTYATL for HLA-A2; RYLRQQLLGI for HLA-A24; RLRDLLLIVTR for HLA-A3 supertype; EVIPMFSAL for HLA-A26). Antigen-specific IFN-γ secretion after an 18 hr incubation was determined by ELISPOT, according to the manufacturer's instructions (MBL, Nagoya, Japan). All assays were carried out in triplicate and analyzed with an ELISPOT reader (CTL-ImmunoSpot S5 Series; Cellular Technology Ltd, Shaker Heights, OH). Antigen-specific T-cell responses were evaluated by the difference between the spot numbers in response to the corresponding peptide and those to the control peptide; differences of threefold were considered significant. If the spot numbers in response to the corresponding peptide in the post-vaccination PBMCs were more than threefold higher than those in the pre-vaccination PBMCs, the changes were considered to be significant. If a significant increase was observed in at least one vaccinated peptide, the specific T-cell response was considered to be augmented.

Statistical Analysis

Demographics were compared between groups using χ^2 test or Fisher's exact test for categorical variables and the Student's *t*-test for continuous variables. Probabilities of progression free survival (PFS) and OS in patients with PPV were estimated from the first date of peptide vaccination to PD or death using Kaplan–Meier methods. In comparison of OS between the patients with PPV with prior DBC and PD patients as the matched control cohort, OS were estimated from the date of PD after DBC to death in order to assess uniformly. All analyses are by intent to treat. Surviving patients were censored at October 7, 2010, with follow-up of 97% complete. Patients lost to follow-up are censored at the date last known to be alive. Cox proportional hazard regression analysis was used to develop the univariate and multivariate models describing the association of the independent variables with OS. Independent variables analyzed included age, EOCG performance status, lymphocyte counts, PSA, CRP, SAA, IL-6, prior DBC status, IgG responses, and T-cell responses. All baseline parameters in the models were analyzed as dichotomous variables using cut-off values. Data were analyzed using GraphicPad Prism software (GraphPad Software, San Diego, CA) and the StatView® program (SAS Institute Inc., Cary, NC). A two-sided significance level of 5% was considered statistically significant.

RESULTS

Patients' Characteristics

Between November 2008 and April 2010, 42 patients with CRPC (20 with prior DBC and 22 without prior DBC) were enrolled into this study. All patients with DBC received 60–75 mg/m^2 docetaxel and repeated every 3–4 weeks in combination with oral prednisone (10 mg/day) or dexamethasone (2 mg/day). Median cycle of docetaxel was 6.5 and failed in treatment. Baseline patient characteristics were well balanced between two study cohorts. No statistically significant differences existed between the groups excluding prior DBC. There was no correlation between Gleason score and pre-vaccine PSA doubling times. Before the enrollment all patients received androgen deprivation therapy using luteinizing hormone-releasing hormone (LH-RH) analogue (or had previously undergone castration) or anti-androgen as an initial or secondary hormonal therapy. All patients were eligible and assessable. The control group at the Dokkyo Medical University Koshigaya Hospital represented 17 patients initiating DBC between September 2007 and August 2010. Table I shows patients' characteristics of the study and control group at the Dokkyo Medical University Koshigaya Hospital. The study cohort with prior DBC and control group were well matched regarding median age, ECOG performance status, and median cycle of DBC (6.5 cycles vs. 7 cycles). The study cohort had a higher level of median PSA levels and higher metastatic sites than the control group.

TABLE I. Patient Demographics and Clinical Characteristics

Characteristics	PPV				Matched control PD after DBC (n = 17)	
	Without prior DBC (n = 22)		With prior DBC (n = 20)			
	No. of Patients	%	No. of Patients	%	No. of Patients	%
Age, years						
Median	70.5		70		71	
Range	53–87		61–81		54–80	
ECOG performance status						
0	22	100	17	85	15	88
1	0	0	3	15	2	12
HLA typing						
A24	16	73	13	65	—	—
A2	4	18	4	20	—	—
A3 super type	2	9	3	15	—	—
PSA, ng/ml						
Median	23.4		87.8		14.7	
Range	0–1,920		4.2–1,508		0.016–317	
PSA doubling time, months						
Median	2.7		3.4		—	—
Range	0.5–36		1.4–60		—	—
Lymphocyte, 1,400 μl^{-1}						
Low	8	36	10	50	—	—
High	14	64	10	50	—	—
CRP, 3,000 ng/ml						
Low	11	50	8	40	—	—
High	11	50	12	60	—	—
SAA, 20,000 ng/ml						
Low	13	59	3	15	—	—
High	9	41	17	85	—	—
IL6, 2 pg/ml						
Low	19	86	15	75	—	—
High	3	14	5	25	—	—
Gleason score						
6	1	4	2	10	0	0
7	6	28	6	30	4	23
8	3	14	1	5	3	18
9	10	46	8	40	8	47
10	1	4	2	10	2	12
Unknown	1	4	1	5	0	0
Site of metastasis						
No	3	14	0	0	4	23
Bone only	7	32	9	45	7	42
Bone and nodal/organ	10	46	9	45	2	12
Nodal/organ	2	8	2	10	4	23
Cycle of DBC						
Median	—		6.5		7	
Range	—		1–27		2–19	

PPV, personalized peptide vaccination; DBC, docetaxel-based chemotherapy; PD, progression disease; ECOG, Eastern Cooperative Oncology Group; HLA, human leicocyte antigen; PSA, prostate-specific antigen; CRP, C reactive protein; SAA, serum amyroid A; IL6, interleukin 6.

Peptides Selection and Immune Responses

Before the peptide vaccination, anti-peptide IgG levels were examined in all 42 patients, and two to four peptides were selected for each patient. The most frequently selected peptides were SART2 $_{161-169}$ (14/42), SART3$_{109-118}$ (13/42), MRP3 $_{503-511}$ (12/42), Lck$_{486-494}$ (9/42), PAP$_{213-221}$ (8/42), HNRPL $_{501-510}$ (8/42), and MRP3 $_{1293-1302}$ (7/42). Lck $_{246-254}$, WHSC2 $_{141-149}$, and SART3 $_{309-317}$ were not selected in this trial.

Both humoral and T-cell responses specific to the vaccinated peptides were analyzed in blood samples before and after the sixth vaccination. Plasma samples were obtained from all patients before and at the time of the sixth vaccination. The post-vaccination samples were not available in one patient with prior DBC, who failed to complete the first cycle of six vaccinations because of disease progression. Table II shows the levels of IgG and T-cell responses in each patient prior to the vaccinations and at the sixth vaccination.

For the monitoring of humoral immune responses, peptide-specific IgG titers were measured by bead-based multiplex assay. The IgG responses specific to at least one of the vaccinated peptides were revealed in 9 of 19 (47%) patients with prior DBC and in 9 of 22 (41%) patients without prior DBC at the 6th vaccination, respectively.

T-cell responses to the vaccinated peptides were measured by IFN-γ ELISPOT assay with PBMCs. PBMCs were available for this assay in 42 and 41 patients before and at the time of the 6th vaccination, respectively. In the pre-vaccination samples, antigen-specific T-cell responses were detectable in 2 of 19 (11%) patients with prior DBC and 5 of 22 (23%) patients without prior DBC, respectively. At the time of the sixth vaccination, T-cell responses to the vaccinated peptides were boosted in 6 of 19 (32%) patients with prior DBC and 8 of 22 (36%) patients without prior DBC. Collectively, antigen-specific T-cell responses were rarely detected in PMBCs before vaccination. In addition, the increase in either peptide-specific IgG titers or T-cell responses at the sixth vaccination was observed in a subset of patients. Notably, the increase in immune responses to each vaccine antigen was not uniformly robust, probably due to the heterogeneity of host immune systems.

Treatment and Efficacy

The median number of vaccinations was 13.5 (range; 5–26) in patients with prior DBC and 14 (range; 6–30) in patients without prior DBC, respectively. One patient with prior DBC did not complete the six scheduled vaccinations because of disease progression. PSA decrease by ≥50% was observed in 15% of the patients with prior DBC and in 9% of the patients without prior DBC. No objective responses were observed in this study. During a median follow-up of 2.7 months, 17 PD occurred in patients with prior DBC; 16 patients had a PSA progression and 1 patient had a new lesion on bone scan, and 16 PD occurred in patients without prior DBC; 14 patients had a PSA progression and 2 patients had a new lesion on bone scan. The median PFS was 2.5 months (95% CI, 1.4–3.6 months) for patients treated by PPV with prior DBC and 2.6 months (95% CI, 0.8–4.4 months) for those treated by PPV without prior DBC (Fig. 1 A). The difference in PFS between the two groups was not significant (log-rank test; $P = 0.48$).

All 42 patients were analyzed for OS with a median follow-up of 11.1 months. At the time of analysis, 15 deaths had occurred; 10 (50%) in PPV with prior DBC and 5 (22.7%) in PPV without prior DBC. Median OS time was 14.8 months (95% CI, 9.7–20.0 months) in patients with prior DBC and not reached in patients without prior DBC within 22.2 months (log-rank; $P = 0.07$) (Fig. 1 B). The hazard ratio (HR) was 0.38 (95% CI, 0.13–1.13; $P = 0.081$) favoring the PPV without prior DBC group.

To assess the usefulness of PPV for patients with prior DBC, we compared the median OS time from the date of PD, after DBC was treated by PPV, with those of historical data in the Dokkyo Medical University Koshigaya Hospital in which patients did not receive PPV but had PD after DBC (n = 17). During a median follow-up of 15.5 months, 19 deaths had occurred; 10 (50%) in PPV with prior DBC and 9 (52.9%) in the historical group. The median OS time was 17.8 months (95% CI, 14.9–20.6 months) in patients with PPV and 10.5 months (95% CI, 7.1–14.0 months) in patients with DBC alone (log-rank; $P = 0.1656$) (Fig. 1C). The OS in the patients treated by PPV with prior DBC seemed to be more favorable than control patients with PD after DBC.

We performed Cox proportional hazard analysis to identify the prognostic factors, which were significantly associated with OS, from clinical findings or laboratory data including age, EOCG performance status, lymphocyte counts, PSA, CRP, SAA, IL-6, prior DBC status, IgG responses, and T-cell responses. As preliminary analysis, a univariate Cox analysis was carried out. IL-6 in pre-vaccine samples was only significantly associated with OS ($P = 0.0012$). None of the other factors studied were significant. Subsequently, multivariate Cox regression analysis was performed to evaluate the influence of each factor on OS after adjusting for possible confounding factors (Table III). The factors showing P less than 0.1 in the univariate analysis including IL6 ($P = 0.0012$), EOCG performance status ($P = 0.0726$), SAA ($P = 0.0632$),

TABLE II. Levels of IgG and T-Cell Responses in 42 CRPC Patients

PPV without prior DBC (n = 22)						PPV with prior DBC					
		IgG response (FIU)		T cell response (pg/ml)				IgG response (FIU)		T-cell response (pg/ml)	
Case	Selected peptide	Pre	6th	Pre	6th	Case	Selected peptide	Pre	6th	Pre	6th
1	Lck-422	1223	2059	—	—	23	SART3-109	548	173	—	—
	ppMAPkkk-432	2893	4710	—	—		MRP3-503	158	133	—	—
	WHSC-103	1351	2513	—	—		PSMA-624	244	140	—	—
	HNRPL-140	145	1689	—	—		EZH2-735	189	132	—	—
2	SART3-109	2066	2158	—	—	24	WHSC-103	226	175	—	—
	PAP-213	1354	1134	—	—		HNRPL-140	161	119	—	—
	PSA-248	7614	7331	—	—		SART3-511	86	62	—	—
	MRP3-503	1560	1522	—	—		SART3-734	71	40	—	—
3	Lck-422	283	274	—	—	25	SART3-109	1132	619	—	—
	SART3-109	501	405	—	—		ppMAPkkk-432	58	58	—	—
	SART2-161	340	408	—	—		HNRPL-501	12	0	—	949
	Lck-486	496	581	—	—		WHSC-103	119	122	—	217
4	SART3-511	363	300	—	—	26	SART2-93	61	51	—	—
	Lck-422	358	269	—	442		SART3-109	702	0	—	—
	ppMAPkkk-432	249	422	—	—		PAP-213	254	143	—	—
	WHSC-103	755	579	—	586		SART2-161	104	76	—	—
5	WHSC-103	376	389	—	—	27	SART3-109	354	202	—	—
	HNRPL-501	359	0	—	—		WHSC2-103	305	398	—	—
	UBE2V-43	855	517	—	—		ppMAPkkk-432	213	265	—	—
	SART3-309	628	647	—	404		HNRPL-501	73	83	—	618
6	MRP3-1293	38	15	—	—		WHSC-103	305	398	—	—
	SART2-161	15	0	—	—	28	HNRPL-501	240	135	—	—
	Lck-486	23	32	—	—		SART3-511	101	0	—	—
7	PAP-213	28	1144	930	1600		SART3-734	73	58	650	—
	PSA-248	97	1119	—	—		Lck-90	46	40	—	418
	MRP3-1293	23	24	567	—	29	UBE2V-43	656	1288	—	—
	Lck-488	31	28	—	—		SART3-302	58	66	—	—
8	MRP3-503	22	27	—	—	30	UBE2V-85	15	31087	—	—
	MRP3-1293	54	59	474	—		MRP3-1293	15	0	—	—
	Lck-488	37	38	446	4514	31	PSA-248	131	30	—	—
	PSMA-624	18	26	484	407		MRP3-503	171	172	—	—
9	Lck-208	164	114	—	—		MRP3-1293	129	0	—	—
	MRP3-503	34	25	—	—		PAP-213	92	13	—	—
10	UBE2V-85	33	24	—	—		SART2-161	112	432	—	—
	EGF-R-800	12	0	—	—	32	HNRPL-501	37	0	—	—
	MRP3-503	47	0	—	757		UBE2V-43	289	12121	—	—
	PTHrP-102	55	110	—	—		UBE2V-85	51	534	—	—
11	EGF-R-800	12	0	—	—		SART3-309	28	15	—	—
	EZH2-735	22	0	—	—	33	SART3-734	166	412	—	—
	PTHrP-102	11	0	—	—		Lck-449	23	0	—	—
	PAP-248	21	0	—	—	34	SART2-93	21	0	—	1667
12	SART3-109	25549	24995	302	—		MRP3-503	54	67	—	1403
	PAP-213	16460	18292	—	—	35	SART2-93	70	86	—	—
	SART2-161	10622	16597	349	428		EGFR-800	122	154	—	—
	PTHrP-102	7929	16617	—	—		SART2-161	144	139	—	—
13	PSA-248	329	373	—	—		EZH2-735	86	192	—	—
	PTHrP-102	251	0	—	—	36	ppMAPkkk-432	262	285	—	—
14	UBE2V-85	141	103	—	—		UBE2V-85	16	13	—	—
	MRP3-503	54	57	—	—	37	PAP-213	45	24	—	—
	SART2-161	72	59	—	—		SART2-161	79	65	—	—
	Lck-486	49	1187	—	—	38	CypB-129	87	82	—	—

(*Continued*)

TABLE II. (*Continued*)

PPV without prior DBC (n = 22)						PPV with prior DBC					
		IgG response (FIU)		T cell response (pg/ml)				IgG response (FIU)		T-cell response (pg/ml)	
Case	Selected peptide	Pre	6th	Pre	6th	Case	Selected peptide	Pre	6th	Pre	6th
15	MRP3-503	11	1361	—	3443		HNRPL-501	97	105	—	3556
	SART2-161	41	77	—	2114		MRP3-503	752	18483	—	1717
16	PAP-213	25	23	—	—	39	SART3-109	2138	NA	—	NA
	MRP3-503	52	41	—	—		PSA-248	16	NA	—	NA
	SART2-161	18	16	—	—		SART2-161	23	NA	—	NA
17	CypB-129	1146	1438	—	—		Lck-486	1085	NA	—	NA
	PAP-213	185	252	—	—	40	SART2-93	77	71	—	—
	SART2-161	29	30	—	—		SART3-109	2904	3360	—	—
	Lck-486	1556	5573	680	—		MRP3-1293	112	0	279	—
18	CypB-129	10	39	—	—		Lck-486	1477	1639	—	—
	HNRPL-501	74	1449	758	14378	41	SART3-109	3273	16554	—	—
	UBE2V-43	20	367	—	2085		PSA-248	29	218	—	—
19	SART3-109	3244	0	—	—		MRP3-503	61	117	—	3457
	SART3-511	234	374	—	—		SART2-161	32	36	—	—
	Lck-90	23	25	—	—	42	SART2-93	31	0	—	—
	Lck-422	66	70	—	—		MRP3-503	13	0	—	—
20	SART2-93	622	0	—	592		SART2-161	50	0	—	454
	SART3-109	15746	162519	—	—		SART3-511	2649	6478	—	—
	Lck-486	4038	4073	—	371						
	Lck-488	2604	2170	—	—						
21	Lck-422	15	0	—	—						
	ppMAPkkk-432	44	0	—	—						
	HNRPL-501	49	0	—	276						
	UBE2V-43	189	0	—	—						
22	SART2-161	15	0	—	—						
	Lck-486	877	859	—	—						
	Lck-488	22	22	—	—						

PPV, personalized peptide vaccination; CRPC, castration-resistance prostate cancer; DBC, docetaxel based chemotherapy; NA, not available.

and prior DBC status ($P = 0.0809$) were included in multivariate analysis of the Cox proportional hazards model. Finally, a lower IL-6 value in pre-vaccine samples from all 42 patients with PPV was a significantly favorable factor for OS ($P = 0.0011$) with a HR of 0.21 (95% CI: 0.068–0.068). However, the other factors had no significant association. In addition, multivariate analysis in DBC-resistant CRP patients similarly showed that a lower IL-6 value was significantly favorable factor for OS ($P = 0.0161$) with a HR of 0.024 (95% CI: 0.001–0.499).

Toxicity

There were no grade 4 toxicities and no treatment-related deaths. The overall toxicities are shown in Table IV. The most frequent adverse events were dermatological reactions at injection sites (n = 39), lymphocytopenia (n = 15), increased AST (n = 12), hypoalbuminemia (n = 11), and bone pain (n = 9). Severe adverse events with grade 3 were as follows: Lymphocytopenia (n = 4), increased AST (n = 2), renal failure (n = 2), bone pain (n = 1). All four patients with severe lymphocytopenia had multiple bone metastasis and progressed during PPV. Lymphocytopenia might be caused by cancer-related bone marrow suppression or immunosuppression. According to the evaluation by the independent safety evaluation committee in this trial, all of these severe adverse events were concluded to be not directly associated with the vaccinations, but with cancer progression or other causes.

DISCUSSION

Although not conclusive due to the small number of patients and the short term of observation in this early phase trial, we demonstrate that PPV is feasible,

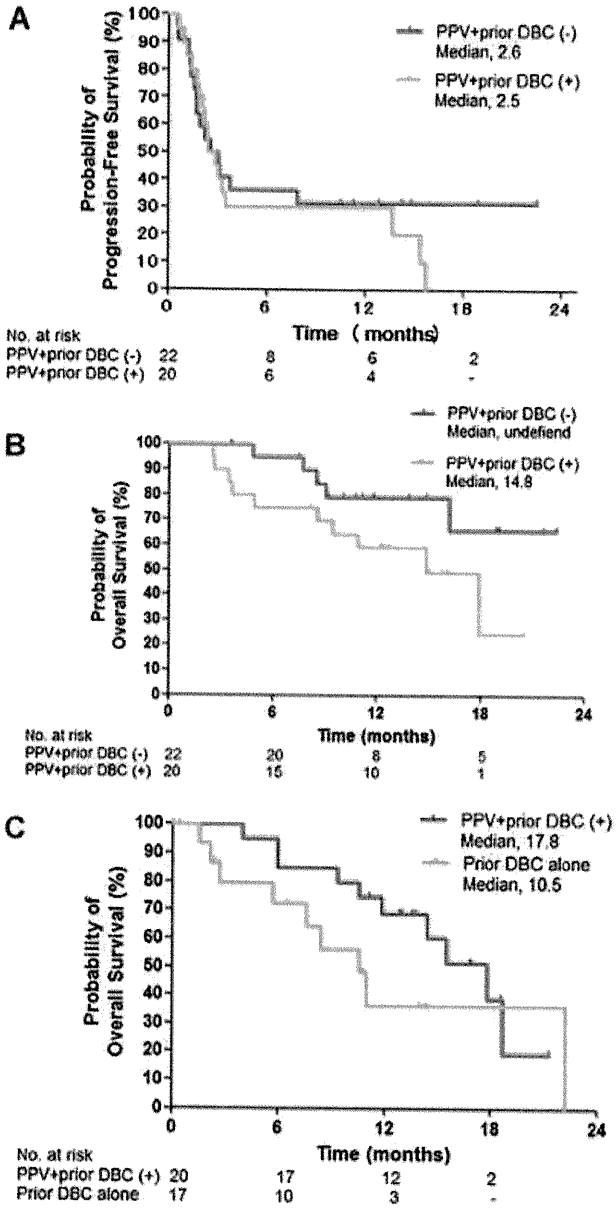

Fig. 1. Kaplan–Meier curves for (**A**) progression-free survival and (**B**) overall survival comparing PPV plus prior DBC(−) with PPV plus prior DBC(+). Kaplan–Meier curves for (**C**) overall survival comparing PPV plus prior DBC(+) with prior DBC alone. PPV, personalized peptide vaccination; DBC, docetaxel-based chemotherapy.

safe, and sufficiently active to induce prolonged OS and immune responses even in patients with PD after DBC. PPV was well tolerated in all patients with CRPC, and most adverse events were grade 1 or 2 local redness and swelling at the injection site. The toxicity reported here was tolerable and considered acceptable in the treatment of the vast majority of metastatic CRPC patients—especially most patients who have a reduced performance status due to the first line DBC, older age, and non-tumor-related infliction.

In this study, median OS time was 14.8 months (95% CI, 9.7–20.0 months) in patients with prior DBC and not reached in patients without prior DBC within 22.2 months (log-rank; $P = 0.07$). The HR was 0.38 (95% CI, 0.13–1.13; $P = 0.081$) favoring the PPV without prior DBC group. Consistent with these findings, our previous studies showed a long survival in CRPC patients without prior DBC by PPV. Results from a phase I and extension study with PPV in CRPC patients without prior DBC (n = 15) showed its safety and the higher frequency of boosting immune responses with a median OS of 23.8 months [15]. Fifty-eight patients with HLA-A2 or HLA-A24 with CRPC without prior DBC were treated with a combination of PPV and low-dose estramustine phosphate (EMP) in a phase I/II study [27]. As a result, the majority (76%) of patients showed a decreased serum PSA level, along with a median OS time of 17 months (95% CI, 12–25 months). In a randomized, cross over, phase II trial of PPV plus low-dose EMP comparing standard-dose EMP in patients with CRPC without prior DBC, the median OS for the PPV plus low-dose EMP group was not reached within 22.4 months and the median OS for the standard-dose EMP group was 16.1 months (95% CI, 8.0–13.4 months) ($P = 0.0328$). The HR for OS was 0.3 in favor of the PPV plus low-dose EMP group. These results suggest that PPV is well tolerated and active in CRPC patients without prior DBC [10].

On the other hand, despite the increasing prevalence of DBC resistant prostate cancer, there are limited studies and no effective treatment in this setting. Briefly, the results of cytotoxic therapy in the second line setting have demonstrated that CRPC in general is poorly controlled after resistance to DBC with a time of progression of 3 months or less with second line therapy and a median OS of approximately 12 months [7,28]. In the current study, the median OS time in CRPC patients with prior DBC was 14.8 months. This result seemed to be a long survival in CRPC patients after PD prior DBC. Since our study was not a randomized phase II study, we attempted to compare our study results to available historical data with similar baseline prognostic features. The OS after PD prior DBC in patients with PPV was improved compared to the Dokkyo Medical University Koshigaya Hospital data. The OS in the patients treated by PPV with prior DBC seemed to be more favorable than control patients with PD after DBC (17.8 vs. 10.5 months, $P = 0.1656$). PPV may have an impact on survival in CRPC patients after PD prior DBC. However, this result was from a retrospective

TABLE III. Cox Proportional Hazards Regression Analysis of Association Between Potential Factors and Death After the PPV in the 42 CRPC Patients

		Univariate			Multivariate		
Factors	Cutoffs[a]	*P*-value	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI
IL6	Low (<2 pg/ml) vs. high	0.0012	0.162	0.054–0.487	0.0075	0.212	0.068–0.661
SAA	Low (<20,000 ng/ml) vs. high	0.0632	0.311	0.091–1.060	0.7596	0.781	0.161–3.788
EOCG performance status	0 vs. 1	0.0726	0.307	0.084–1.115	0.3851	0.526	0.124–2.242
Prior DBC status	Untreated vs. treated	0.0809	0.380	0.128–1.126	0.4026	0.573	0.156–2.110
PSA	Low (<40 ng/ml) vs. high	0.2751	0.548	0.174–1.613	—	—	—
Pts. Age	Low (<70 years) vs. high	0.2853	0.569	0.202–1.603	—	—	—
Number of lymphocytes	High (>1,400 μl^{-1}) vs. low	0.3383	0.609	0.220–1.681	—	—	—
T-cell response	Positive vs. negative	0.4694	0.654	0.207–2.066	—	—	—
CRP	Low (<3,000 ng/ml) vs. high	0.6543	0.790	0.282–2.217	—	—	—
IgG response	Positive vs. negative	0.8900	1.088	0.329–3.597	—	—	—

Of the 42 men 19 had death.
PPV, personalized peptide vaccination; CRPC, castration-resistance prostate cancer; CI, confidence intervals; DBC, docetaxel-based chemotherapy; ECOG, Eastern Cooperative Oncology Group; PSA, prostate-specific antigen; CRP, C reactive protein; SAA, serum amyroid A; IL6, interleukin 6.
[a]Lymphocyte, PSA and patient age are based on median values.

analysis comparing historical data. Randomized trials with an appropriate control group based on survival as the primary end point of efficacy should be required to identify this result.

In contrast to OS, the time to disease progression as defined in this study was short and did not differ significantly between the study groups. This result may be due to the delayed onset of anti-tumor responses after active immunotherapy, relative to disease progression, which occurred early in this group of patients [29]. In patients with metastatic CRPC, the disease-progression end point has not been a reliable predictor of OS. Several randomized trials that have shown effects of various treatments on OS have not shown effects on disease progression [30,31].

Cancer vaccinations do not elicit beneficial immune and/or clinical responses in all of the treated patients. Therefore, identification of surrogate biomarkers for predicting immune and/or clinical responses in vaccinated patients would be an important, but challenging issue allowing for individualized therapy. At present, however, there has been little information available regarding the predictive biomarkers identified in patients undergoing cancer vaccinations. Chronic inflammation is a key contributor to cancer development and progression [32]. Cancer survivors with chronic inflammation may have an elevated risk of recurrence as a result of the effects of inflammatory processes on cell growth or the presence of cancer cells that induce inflammation.

TABLE IV. Adverse Events

	G1	G2	G3	G4	Total
Injection site reaction	5	34	0	0	39
Lymphocytopenia	5	6	4	0	15
AST increased	12	0	2	0	14
Anemia	3	8	1	0	12
Hypoalbuminemia	7	4	0	0	11
Bone pain	2	5	2	0	9
Fatigue	2	5	0	0	7
Appetite loss	0	5	0	0	5
ALT increased	5	0	0	0	5
Blood triglycerides increased	5	0	0	0	5
Oedema peripheral	0	3	0	0	3
Renal failure	0	0	2	0	2
White blood cell count decreased	2	0	0	0	2

Elevated CRP has been associated with poor survival in metastatic prostate [33] and other cancers [34,35]. Preoperative SAA has been associated with survival in gastric cancer and renal cell carcinoma patients [36,37]. Similarly, elevated IL-6 have been associated with features of aggressive cancer and decreased survival in prostate cancer patients [38]. In this respect, we investigated whether CRP, SAA or IL-6 are predictive biomarkers for OS. Interestingly, one of the most important findings in this current study is that lower levels of IL-6 in pre-vaccine samples was significantly favorable factors for OS in the univariate and multivariate analysis. This finding suggested that this inflammatory molecule may potentially act as a surrogate biomarker for predicting a poor prognosis in patients with CRPC undergoing PPV. IL-6 is a multifunctional cytokine that regulates various aspects of the immune responses, acute phase reactions, and hematopoiesis. In particular, IL-6 has recently been reported to be one of the critical cytokines for inducing suppressive immune cell subsets [35–37]. For example, Myeloid-derived suppressive cells (MDSCs), which are known to suppress anti-tumor immunity, were shown to be rapidly generated from precursors present in murine and human bone marrow or PBMCs in the presence of IL-6 and other cytokines, such as GM-CSF [39,40]. Another combination of cytokines, IL-6 and TGF-β, were also reported to induce a recently identified subset of helper T cells, Th17, which may promote cancer progression [41–43]. Although the precise role of IL-6 in immune responses to cancer vaccines remains to be clarified, modulation or blockage of IL-6 signaling may provide benefits in patients undergoing PPV.

In conclusion, this study showed that PPV is well tolerated, and although limited responses were observed, it may have an impact on survival in CRPC patients with PD after DBC in a retrospective analysis. These encouraging preliminary results suggested that PPV warrants further study as a novel therapy for CRPC patients with PD after DBC. Importantly, this study includes an evaluation of IL-6 as an efficacy biomarker for OS in CRPC patients treated by PPV. IL-6 may potentially act as a surrogate biomarker for predicting a poor prognosis in patients with CRPC undergoing PPV, and warrants further investigation.

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Original Article

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

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BACKGROUND: Because only a subset of patients show clinical responses to peptide-based cancer vaccination, it is critical to identify biomarkers for selecting patients who would most likely benefit from this treatment. **METHODS:** The authors characterized the gene expression profiles in peripheral blood of vaccinated patients to identify biomarkers to predict patient prognosis. Peripheral blood was obtained from advanced castration-resistant prostate cancer patients, who survived for >900 days (long-term survivors, n = 20) or died within 300 days (short-term survivors, n = 20) after treatment with personalized peptide vaccination. Gene expression profiles in prevaccination and postvaccination peripheral blood mononuclear cells (PBMCs) were assessed by DNA microarray. **RESULTS:** There were no statistically significant differences in the clinical or pathological features between the 2 groups. Microarray analysis of prevaccination PBMCs identified 19 genes that were differentially expressed between the short-term and long-term survivors. Among the 15 up-regulated genes in the short-term survivors, 13 genes, which were also differentially expressed in postvaccination PBMCs, were associated with gene signatures of granulocytes. When a set of 4 differentially expressed genes were selected as the best combination to determine patient survival, prognosis was correctly predicted in 12 of 13 patients in a validation set (accuracy, 92%). **CONCLUSIONS:** These results suggested that abnormal granulocytes present in the PBMC faction may contribute to poor prognosis in advanced prostate cancer patients receiving personalized peptide vaccination. Gene expression profiling in peripheral blood might thus be informative for devising better therapeutic strategies by predicting patient prognosis after cancer vaccines. ***Cancer*** **2011;000:000–000.** *© 2011 American Cancer Society.*

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS

Patients

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

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

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

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

Blood Samples

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

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

RNA Isolation From PBMCs

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

DNA Microarray Analysis

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

Table 1. Patient Characteristics (Postvaccination Analysis)

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

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

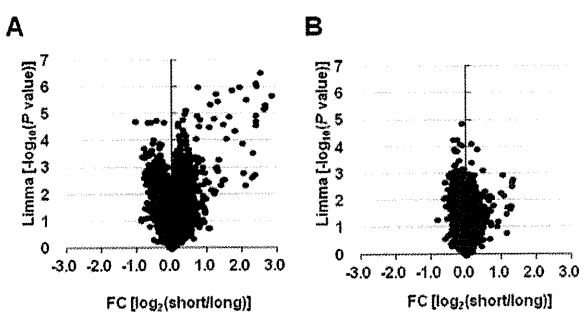

Figure 1. Volcano plots present the microarray data in prevaccination and postvaccination peripheral blood mononuclear cells (PBMCs). The plot graphs the fold-change (FC; $\log_2$[short/long]) on the x-axis versus statistical significance (minus $\log_{10}$ *P* value) on the y-axis in PBMCs (A) after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

qRT-PCR

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

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

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

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

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

Measurement of Soluble Factors in Plasma

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

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

Statistical Analysis

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

RESULTS

Patients

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

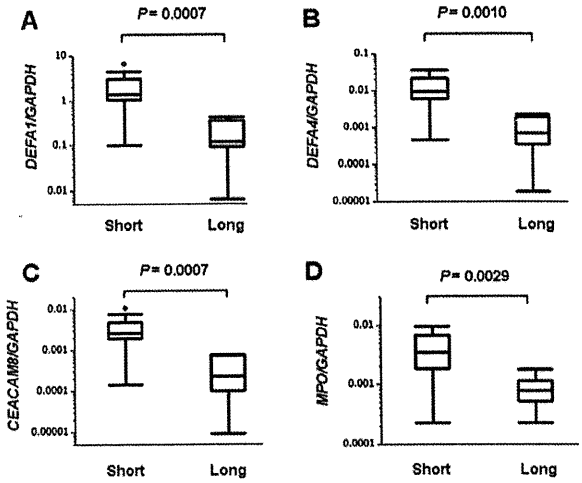

Figure 2. Differential gene expression was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). The gene expression of (A) *DEFA1*, (B) *DEFA4*, (C) *CEACAM8*, and (D) *MPO* were measured by qRT-PCR in postvaccination peripheral blood mononuclear cells of the short-term (n = 12) and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

Identification of Differentially Expressed Genes in Postvaccination PBMCs

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

Table 3. Patient Characteristics (Prevaccination Analysis)

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

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

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

Identification of Differentially Expressed Genes in Prevaccination PBMCs

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

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

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

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

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

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

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

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

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

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

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

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

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).

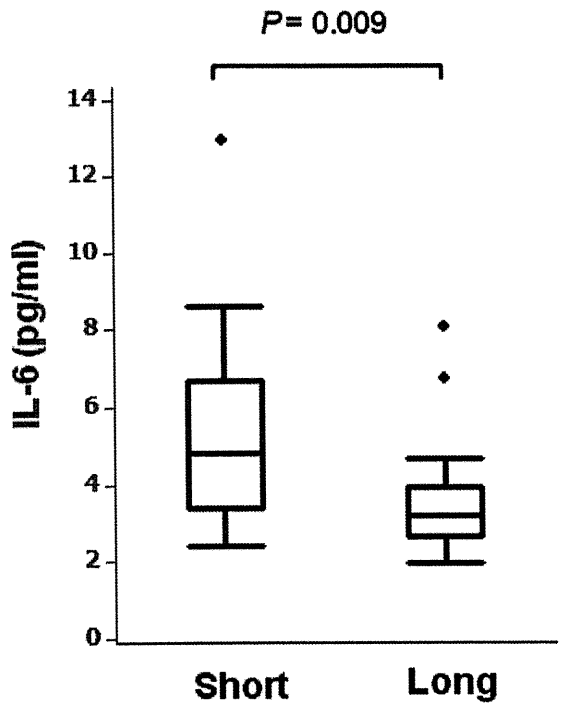

Figure 3. Increase in plasma interleukin (IL)-6 levels in the short-term survivors is shown. The levels of IL-6 assessed by bead-based multiplex assay in prevaccination plasma were compared between the short-term (n = 18) and long-term (n = 18) survivors. Box plots show median and interquartile range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