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,

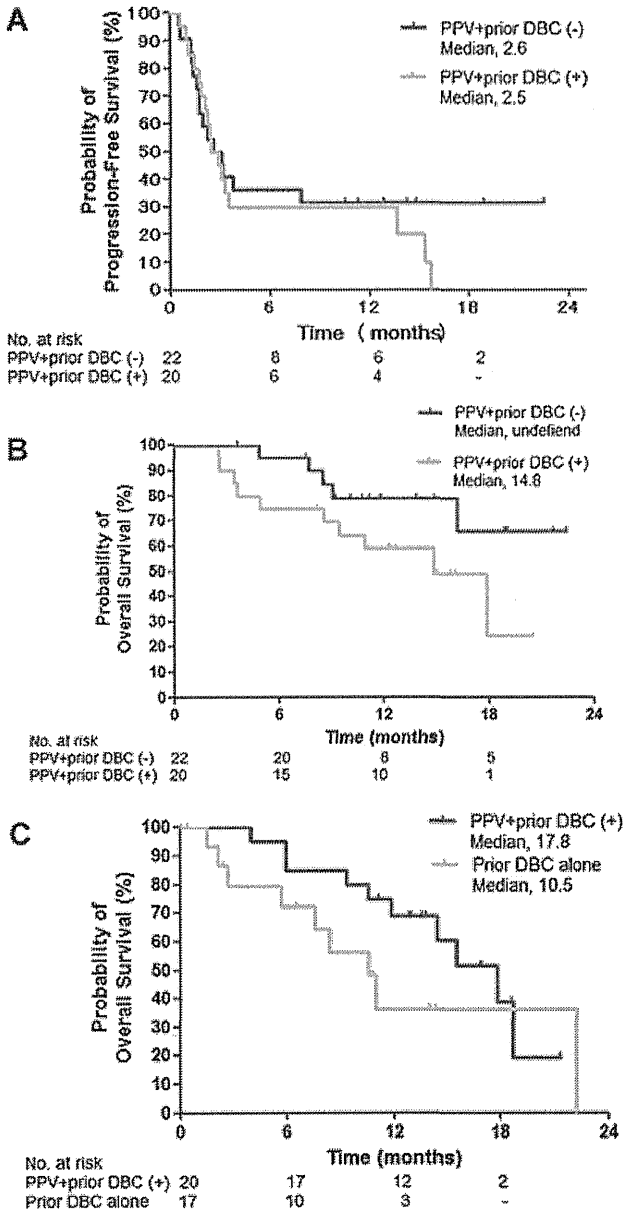

Fig. 1. Kaplan–Meier curves for (**A**) progression-free survival and (**B**) overall survival comparing PPV plus prior DBC(−) with PPV plus prior DBC(+). Kaplan–Meier curves for (**C**) overall survival comparing PPV plus prior DBC(+) with prior DBC alone. PPV, personalized peptide vaccination; DBC, docetaxel-based chemotherapy. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/pros]

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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Personalized peptide vaccination

A novel immunotherapeutic approach for advanced cancer

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Since both tumor cells and immune cell repertoires are diverse and heterogeneous, immune responses against tumor-associated antigens might be substantially different among individual patients. Personalized selection of right peptides for individuals could thus be an appropriate strategy for cancer vaccines. We have developed a novel immunotherapeutic approach, personalized peptide vaccination (PPV), in which HLA-matched peptides are selected and administered, based on the pre-existing host immunity before vaccination. Recent clinical trials of PPV have demonstrated a feasibility of this new therapeutic approach in various types of advanced cancers. For example, a randomized phase II trial for patients with castration resistant prostate cancer showed a possible clinical benefit in the PPV group. In the patients undergoing PPV, lymphocyte counts, increased IgG responses to the vaccine peptides, and inflammatory factors in pre-vaccination peripheral blood might be potential biomarkers for prognosis. Further randomized phase III trials would be recommended to prove clinical benefits of PPV.

Keywords: peptide vaccine, personalized vaccine, cytotoxic T lymphocytes, advanced cancer, biomarker, inflammation

Abbreviations: PPV, personalized peptide vaccination; CTL, cytotoxic T lymphocytes; CRPC, castration-resistant prostate cancer; FDA, food and drug administration; MST, median survival time; HR, hazard ratio; CI, confidence interval

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Introduction

The field of cancer immunotherapy has drastically moved forward during these two decades since Boon and his colleagues reported for the first time a tumor-associated antigen, MAGE-A1, recognized by cytotoxic T lymphocyte (CTL) in 1991.[1] In particular, there have recently been noteworthy advances in the clinical application of cancer immunotherapy.[2,3] In 2010, sipuleucel-T (Provenge; Dendreon Corporation), an autologous cellular immunotherapy product designed to stimulate T cell immune responses against human prostatic acid phosphatase (PAP), was first approved for patients with castration-resistant prostate cancer (CRPC) by the US. Food and Drug Administration (FDA).[4] In addition, another immunotherapeutic agent, ipilimumab, an anti-cytotoxic T lymphocyte antigen (CTLA)-4 monoclonal antibody, was also approved for melanoma patients by the FDA in 2011.[5] Despite these significant advances, however, most of other randomized clinical trials in cancer immunotherapy have so far failed to show beneficial therapeutic effects compared with existing treatments.[6,7] The failure of recent clinical trials has raised several issues to be addressed for development of cancer vaccines. Here, we have proposed a novel immunotherapeutic approach, "personalized peptide vaccination (PPV)" for advanced cancer patients.

Rationale for Personalized Selection of Vaccine Antigens in Individual Cancer Patients

A large number of tumor-associated antigens have been identified by several different approaches, including cDNA expression cloning, serologic analysis of recombinant cDNA expression libraries (SEREX), and reverse immunological approach.[8] Although the number of cancer vaccine candidates is becoming almost limitless, antigens currently employed

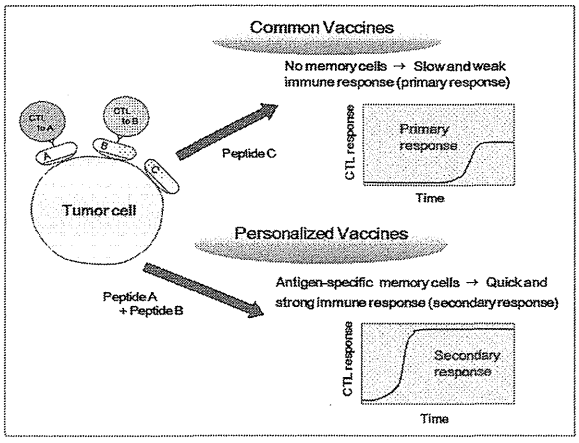

Figure 1. Personalized vaccines are more promising than common vaccines. Personalized antigens can induce quick and strong secondary immune responses, whereas common antigens without immunological memory induce slow and weak primary immune responses.

for vaccination against individual cancer patients might not always be appropriate. In general, anti-tumor immunity is known to be dependent on both immunological characters of tumor cells and immune cell repertoires. Since immune cell repertoires are quite diverse and heterogeneous, anti-tumor immunity might be substantially different among individuals. Therefore, it is likely that vaccine antigens that are selected and administered without considering the immune cell repertoires of the hosts could not efficiently induce beneficial anti-tumor immune responses. To increase the clinical benefits from cancer vaccines, particular attentions should be paid to immunological status of each patient by characterizing the pre-existing immune responses to vaccine antigens before vaccination.

Nevertheless, in most of current clinical trials of therapeutic cancer vaccines, common antigens are employed for vaccination independently of immunological status of patients. Patients, who have immunological memory to vaccine antigens, are expected to show quick and strong immune responses to them. In contrast, patients with no immunological memory against vaccine antigens would take more time for development of effective anti-tumor immune responses, because several rounds of repeated vaccinations might be required to prime antigen-specific naive T cells to functional effector cells (**Fig. 1**). In such situations, vaccinations could not easily provide clinical benefits, especially in advanced cancer patients, who show a relatively quick disease progression. Moreover, immune responses induced by inadequate vaccines that are non-specific to tumor cells may not only be ineffective for tumor control, but also erode pre-existing immunity.[9] Based on the current paradigm that the size and composition of the adaptive immune system are limited and that individual immune cells are constantly competing each other in the limited space, inadequate vaccination may have negative consequences for the hosts by suppressing pre-existing beneficial memory cells specific to tumors and/or infections, which might result in acceleration of cancer progression or early death in vaccinated patients.[10] Considering these issues, it would be quite reasonable that vaccine antigens should be selected based on the pre-existing immunological status in each patient.

In addition, it should be noted that cancer cells possess or develop a variety of mechanisms to maintain their malignant behavior. For example, it has been well recognized that cancer cells escape from host immunological surveillance.[11] Through the interaction between host immune system and tumor cells at the equilibrium phase, immunological pressure often produces tumor cell variants that decrease or lose tumor-associated antigens. Therefore, to better control cancer cells, it would be recommended to target multiple tumor-associated antigens to reduce the risk of outgrowth of antigen-loss variants.

PPV as a Novel Immunotherapeutic Approach

In view of complexity and diversity of immunological characters of tumors and immune cell repertoires, we have developed a new concept of PPV.[12] In this "personalized" cancer vaccine formulation, appropriate peptide antigens for vaccination are screened and selected from a list of vaccine candidates in each patient, based on pre-existing host immunity. Currently, we employ 31 HLA class I-restricted peptide candidates, which were identified from a variety of tumor-associated antigens mainly through cDNA expression cloning method with tumor-infiltrating lymphocyte clones/lines; 12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for HLA-A3 supertype (A3, A11, A31 or A33), and 4 peptides for HLA-A26. The safety and potential immunological effects of these vaccine candidates have been shown in previously conducted clinical studies.[12-14] A maximum of 4 peptides, which are selected based on the results of HLA typing and the pre-existing immune responses specific to each of the 31 different vaccine candidates, are subcutaneously administered in complex with incomplete Freund's adjuvant weekly or bi-weekly.

Currently, we evaluate the pre-existing immune responses to vaccine candidates by B cell responses, but not by T cell responses, since the performance characteristics, such as sensitivity and reproducibility, of current T cell assays are unsatisfactory.[3,15] In contrast to these drawbacks inherent to T cell assays, B cell assays have more potential for screening and/or monitoring antigen-specific immune responses even to MHC class I-restricted peptides. Indeed, we have recently published several papers describing the clear correlations between clinical benefits and antigen-specific B cell responses measured by IgG antibody production in patient plasma after vaccination.[16] Notably, the multiplex bead-based LUMINEX technology that we have developed for monitoring B cell

Table 1. Clinical responses of advanced cancer patients treated with PPV

	Patient (n)	Evaluable patient (n)	Best clinical response (n)			Response rate (%)	Disease control rate (%)
			PR	SD	PD		
Total	500	436	43	144	249	9.9	42.9
Prostatic	174	155	29	36	90	18.7	41.9
Colorectal	74	68	1	23	44	1.5	35.3
Pancreatic	50	41	4	23	14	9.8	65.9
Gastric	42	35	0	8	27	0	22.9
Brain	33	30	5	11	14	16.7	53.3
Cervical	28	23	3	7	13	13.0	43.5
Non-small cell lung	22	21	0	11	10	0	52.4
Renal cell	13	12	0	9	3	0	75.0
Melanoma	12	11	0	5	6	0	45.5
Breast	11	10	0	1	9	0	10.0
Uroepithelial	10	7	1	2	4	14.3	42.9
Others	31	23	0	8	15	0	34.8

Best clinical responses were evaluated by RECIST criteria (or PSA values in prostatic cancer). PR, partial response; SD, stable disease; PD, progressive disease.

responses allows simple, quick and highly reproducible high-throughput screening of IgG responses specific to large numbers of peptide antigens with a tiny amount of plasma.[17]

In the clinical trials of PPV conducted during the past several years, we have shown promising results in various types of cancers.[12,13,16,18,19] Table 1 shows the clinical responses in 500 advanced cancer patients who received PPV from October 2000 to October 2008.[16] The best clinical response assessed in 436 evaluable patients were partial response (PR) in 43 patients (10%), stable disease (SD) in 144 patients (33%) and progressive disease (PD) in 249 patients (57%), with a median overall survival of 9.9 mo. Of note, as shown in Figure 2, a recently conducted phase II randomized clinical trial of PPV for 57 CRPC patients demonstrated that patients receiving PPV in combination with low-dose estramustine phosphate (EMP) showed a significantly longer progression-free [median survival time (MST), 8.5 vs. 2.8 mo; hazard ratio (HR), 0.28 (95% confidence interval (CI), 0.14–0.61); p = 0.0012] and overall survival [MST, undefined vs. 16.1 mo; HR, 0.30 (95% CI, 0.10–0.91); p = 0.0328] than those receiving standard-dose EMP alone.[18] In addition, PPV was also conducted in an early phase clinical trial of patients with recurrent or progressive glioblastoma multiforme, one of the most aggressive brain tumors, with median overall survival of 10.6 mo.[19] Based on these promising results, randomized phase III trials are currently underway in CRPC and glioblastoma. To prove clinical benefits of PPV for accelerating cancer vaccine development, further randomized phase III trials would also be recommended in other different types of cancers.

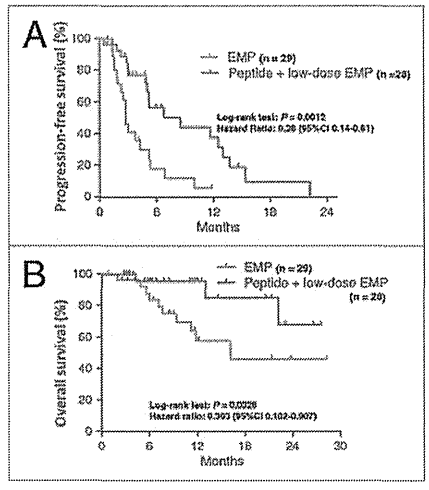

Figure 2. Progression-free and overall survival in patients with castration-resistant prostate cancer using personalized peptide vaccination. Kaplan-Meier curves of progression-free (A) and overall survival (B) in patients treated with personalized peptide vaccination plus low-dose estramustine phosphate (EMP) or standard-dose EMP. Adapted from Noguchi et al.[18]

Lymphocyte Counts, Increased Humoral Responses to the Vaccine Antigens, and Inflammatory Factors as a Biomarker for PPV

Only a subset of patients show clinical benefits from cancer immunotherapy, including peptide-based cancer vaccines. In addition, even worse, some large clinical trials in the past several years

have demonstrated that cancer vaccines might sometimes show worse clinical outcomes.[6,7] Therefore, it would be critical to identify biomarkers that accurately portray anti-tumor immune responses and predict prognosis in treated patients.[3,6] With regard to post-vaccination biomarkers, several factors, including CTL responses, Th1 responses, delayed-type hypersensitivity (DTH) and autoimmunity, have been reported to be associated with clinical responses in some clinical trials.[20-23] However, as they have not been always reproducible in other studies, there are currently no validated prognostic or predictive biomarkers in widespread use.

We also investigated immunological biomarkers in 500 advanced cancer patients who received PPV from October 2000 to October 2008.[16] By the statistical analysis in this patient population, both lymphocyte counts prior to the vaccination ($p = 0.0095$) and increased IgG responses ($p = 0.0116$) to the vaccine peptides, along with performance status ($p < 0.0001$), were well correlated with overall survival.

To identify biomarkers useful for selecting appropriate patients before vaccination, we further addressed pre-vaccination prognostic markers in patients with several different types of advanced cancers who underwent PPV. In CRPC treated with PPV (n = 40), a comprehensive study of soluble factors and gene expression profiles by microarray analysis demonstrated that higher IL-6 level and granulocytic myeloid-derived suppressor cells (MDSC) in the peripheral blood before vaccination were closely associated with poorer prognosis.[24] In patients with refractory non-small cell lung cancer (n = 41), multivariate Cox regression analyses showed that higher C-reactive protein (CRP) level before vaccination was a significant predictor of unfavorable overall survival (HR = 10.115, 95% CI = 2.447–41.806, $p = 0.001$).[25] In addition, in refractory biliary tract cancer patients (n = 25), higher IL-6 and lower albumin levels before vaccination were significantly unfavorable factors for overall survival [HR = 1.123, 95% CI = 1.008–1.252, $p = 0.035$; HR = 0.158, 95% CI = 0.029–0.860, $p = 0.033$; respectively].[26] Collectively, these findings have demonstrated that less inflammation may contribute to better responses to PPV, suggesting that evaluation of the inflammatory factors before vaccination could be useful for selecting appropriate cancer patients for PPV. Based on these findings, an early phase clinical trial is currently underway to show whether the blockage of IL-6-mediated inflammatory signaling with a humanized anti-IL-6 receptor monoclonal antibody, tocilizumab, would be beneficial for enhancing the immune and/or clinical responses of PPV.[27]

Conclusions

The field of cancer immunotherapy has drastically moved forward during the past 20 years, but there have been several issues to be addressed for success of cancer vaccine development. In view of complexity and diversity of immunological characters of tumors and immune cell repertoires, we have developed a new concept of PPV. In the clinical trials conducted during the past several years, we have shown promising results of PPV as a new treatment modality for patients with various types of advanced cancers. Further randomized phase III clinical trials would be essential to prove clinical benefits of PPV. In addition, novel biomarkers for selecting patients who would most benefit from PPV remain to be identified.

Disclosure of Potential Conflicts of Interest

The authors have no conflict of interest and financial relationships to disclose.

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Original Article

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

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

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

INTRODUCTION

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

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

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

MATERIALS AND METHODS

Patients

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

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

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

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

Blood Samples

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

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

RNA Isolation From PBMCs

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

DNA Microarray Analysis

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

Table 1. Patient Characteristics (Postvaccination Analysis)

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

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

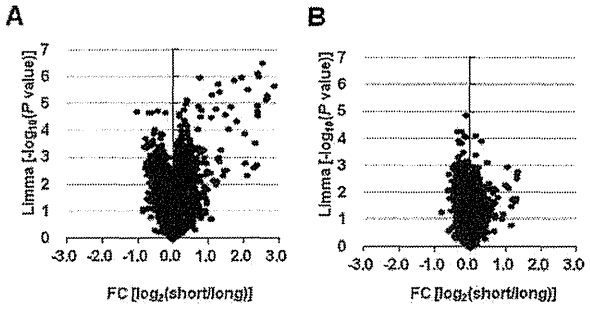

Figure 1. Volcano plots present the microarray data in prevaccination and postvaccination peripheral blood mononuclear cells (PBMCs). The plot graphs the fold-change (FC; $\log_2$[short/long]) on the x-axis versus statistical significance (minus $\log_{10}$ *P* value) on the y-axis in PBMCs (A) after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

qRT-PCR

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

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

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

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

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

Measurement of Soluble Factors in Plasma

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

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

Statistical Analysis

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

RESULTS

Patients

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

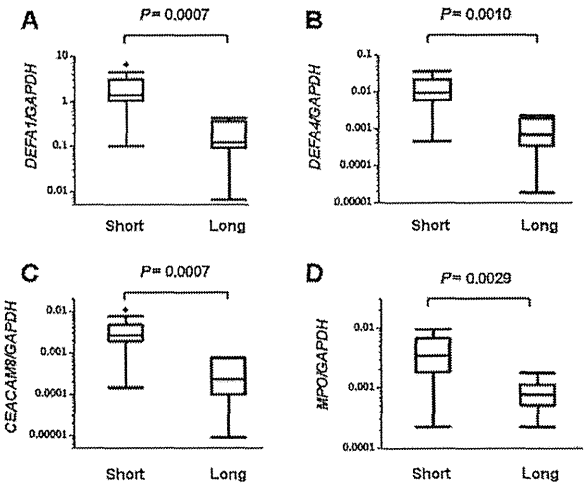

Figure 2. Differential gene expression was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). The gene expression of (A) *DEFA1*, (B) *DEFA4*, (C) *CEACAM8*, and (D) *MPO* were measured by qRT-PCR in postvaccination peripheral blood mononuclear cells of the short-term (n = 12) and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

Identification of Differentially Expressed Genes in Postvaccination PBMCs

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

Table 3. Patient Characteristics (Prevaccination Analysis)

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

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

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

Identification of Differentially Expressed Genes in Prevaccination PBMCs

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

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

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

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

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

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

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

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