used the data from a total of 500 advanced cancer patients, who received personalized peptide vaccination conducted between October 2000 and October 2008, to investigate biomarkers that are predictive of their overall survival. Furthermore, we used samples from long-term survivors (more than 900 days of overall survival) and short-term survivors (less than 300 days of overall survival) with advanced castration-resistant prostate cancer (CRPC) under treatment with personalized peptide vaccination. It is well known that advanced CRPC patients rarely survive more than 2 years even if they receive global standard chemotherapy combined with hormone therapy.[12] Therefore, although only 43 patients were examined in subgroup analysis, the clinical benefits in the long-term survivors should be sufficiently large for the statistical analysis to identify definite biomarkers easily if any.

Results

Patient characteristics, immunological and clinical responses. The demographic, immunological responses and clinical characteristics of the 500 patients with advanced cancer are listed in **Table 1A and B**. The most frequent symptom of toxicity in the personalized peptide vaccination was a local skin reaction at injection sites. These symptoms were manageable through routine interventions as reported previously.[13-29] The best response to the personalized peptide vaccination was assessed in 436 patients. No complete responses (CR) were observed in either group. Forty-three patients (10%) had partial response (PR) and 144 patients (33%) had stable disease (SD). The remaining 249 patients (57%) had progressive disease (PD) without responses. Most of these clinical responses were already reported.[13-29] The response rate and disease control rate during the personalized peptide vaccination were 9.9 and 42.9%, respectively.

Correlation between overall survival and immune responses. The median follow-up for all 500 patients was 9.1 months (range, 1–105 months). Forty-five patients (9%) were alive at the end of the study (October 2009). Four hundred and forty-five patients died from advanced cancer and 10 patients died of other causes. The median overall survival time was 9.9 months with 1- and 3-year survival rates of 43 and 10.7%, respectively (**Fig. 1A**). Peptide-specific cellular and humoral immune activities were measured at 6-week intervals as long as patient samples were available. The total numbers of evaluable patients for CTL and IgG responses during the personalized peptide vaccination were 332 and 300, and positive results in CTL and IgG responses after the sixth vaccination were detected in 199 (60%) patients and in 187 (62%) patients, respectively. The median overall survival for patients with a positive IgG response was significantly longer than that for patients with a negative IgG response ($p = 0.0015$ by log-rank test; **Fig. 1C**), while an association between CTL response status and overall survival was not observed ($p = 0.167$ by log-rank test; **Fig. 1B**).

Analysis of predictors of overall survival. Cox proportional hazards regression analysis was performed to determine factors that are predictive of overall survival in the 500 patients listed above (**Table 2**). In univariate regression analysis, performance status ($p < 0.0001$), counts of lymphocytes ($p < 0.0001$), IgG response and age ($p = 0.002$) were found to be associated with survival. Gender, CTL response, HLA typing and vaccine interval were not significant factors. Forward stepwise multivariate analysis showed that only performance status ($p < 0.0001$; hazard risk 2.295; 95% CI, 1.653–3.188), counts of lymphocytes ($p = 0.0095$; hazard risk 1.472; 95% CI, 1.099–1.972) and IgG response ($p = 0.0116$; hazard risk 1.455; 95% CI, 1.087–1.948) were independent predictors of overall survival. None of the other variables were significant predictors of overall survival.

Comparison of immune responses between short- and long-term survivors. To statistically confirm the superiority of IgG response as a predictor to CTL response, samples from 20 patients who survived more than 900 days (long-term survivors) and those from 23 patients who died within 300 days (short-term survivors), among 174 patients with CRPC who received personalized peptide vaccination, were analyzed further. There were no statistical differences between the two groups with regard to clinical and pathological characteristics at the time of entry (**Table 3**). The only apparent difference was overall survival after the vaccination. Median survival times of long- and short-term survivors used for the analysis were 1,483 days and 189 days, respectively.

The frequencies of selection of each peptide candidate at the first vaccination between long- and short-term survivors were investigated to address if the peptides used were different between the two groups. There were no significant differences in the frequencies of selection of each peptide at the first vaccination between the two groups.

The levels of IgG reactive to each of the vaccinated peptides were measured for 21 of 23 short-term survivors and all 20 long-term survivors during both pre-vaccination and post-vaccination periods, and the representative results were given in **Table 4A and B**. The post-vaccination samples were not available from two short-term survivors. In short-term survivors, the numbers of peptides, against which a more than two-fold increase in IgG was observed, were 0 peptide in 10 patients, 1 peptide in 7 patients, 2 peptides in 3 patients and 3 peptides in 1 patient. In long-term survivors, numbers of peptides, to which increased IgG responses were observed, were 0 peptide in 3 patients, 1 peptide in 3 patients, 2 peptides in 5 patients, 3 peptides in 6 patients and 4 peptides in 3 patients ($p = 0.000282$). To better represent n-fold increase in IgG levels, the results were drawn in **Figure 2**, in which the vertical bars denote log 10 scores. In short-term survivors, the numbers of peptides, against which a more than 10-fold increase in IgG was observed, were 0 peptide in 16 patients, 1 peptide in 2 patients, 2 peptides in 2 patients and 3 peptides in 1 patient. In long-term survivors, the numbers of peptides, against which a more than 10-fold increase in IgG was observed, were 0 peptide in 5 patients, 1 peptide in 6 patients, 2 peptides in 5 patients and 3 peptides in 4 patients ($p = 0.00045$).

CTL activity against each of the vaccinated peptides was measured in 17 of 23 short-term survivors and all 20 long-term survivors during both pre-vaccination and post-vaccination periods (**Table 4A and B**). The post-vaccination peripheral blood mononuclear cells (PBMCs) needed for measurement of CTL responses were not available from 9 short-term survivors primarily because of rapid progression of cancer. In short-term survivors, the numbers

Table 1A. Characteristics, Immune responses and clinical responses of 500 patients with advanced cancer

Characteristics	Total		Groups of cancer											
			Prostatic cancer		Colorectal cancer		Pancreatic cancer		Gastric cancer		Brain tumor		Cervical cancer	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
No. of patients	500		174	35	74	15	50	10	42	8	33	7	28	6
Average Age, years	61.8		67.9		58.5		64.8		58.7		49.6		49.9	
Standard deviation	12.8		7.8		12.3		8.8		12.3		20.3		12.4	
Sex														
Male	353	71	174	100	52	70	32	64	29	69	18	55	-	
Female	147	29	-		22	30	18	36	13	31	15	45	28	100
Performance status (ECOG)														
0	333	67	144	83	47	64	33	66	20	48	7	21	16	57
1	118	24	25	14.5	23	31	16	32	16	38	6	19	9	32
2	31	6	1	0.5	4	5	0	0	6	14	8	24	3	11
3	18	3	4	2	0	0	1	2	0	0	12	36	0	0
Peptides bind for HLA														
A2	139	28	48	28	16	22	15	30	14	33	8	24	11	39
A24	332	66	109	63	58	78	31	62	28	67	25	76	17	61
A3-supretype	6	1	4	2	0	0	0	0	0	0	0	0	0	0
Mixed type	23	5	13	7	0	0	4	8	0	0	0	0	0	0
Average times of vaccination	14.7		17		13.9		16.3		9.8		13.1		14	
Standard deviation	15		18.9		11.8		14.3		9.8		11.2		9.9	
Treatment														
Vaccination alone	331	66	109	63	47	64	11	22	34	81	14	42	28	100
Combination	169	34	65	37	27	36	39	78	8	19	19	58	0	0
CTL response														
No. of evaluable case	332		111		60		40		25		28		20	
yes	199	60	75	68	32	53	26	65	15	60	17	65	13	65
no	133	40	36	32	28	47	14	35	10	40	9	35	7	35
IgG response														
No. of evaluable case	300		105		48		41		21		22		12	
yes	187	62	77	73	27	56	21	51	14	67	11	50	7	58
no	113	38	28	27	21	44	20	49	7	33	11	50	5	42
Best clinical response														
No. of evaluable case	436		155		68		41		35		30		23	
PR	43	10	29	19	1	1	4	10	0	0	5	16	3	13
SD	144	33	36	23	23	34	23	56	8	23	11	37	7	30
PD	249	57	90	58	44	65	14	34	27	77	14	47	13	57
Response rate (%)	9.9		18.7		1.5		9.8		-		16.7		13	
Disease control rate (%)	42.9		41.9		35.3		65.9		22.9		53.3		43.5	

Immunological responses were evaluated using the pre-and post-sixth vaccination samples.

of peptides, against which increased CTL responses were observed, were 0 peptide in 4 patients, 1 peptide in 6 patients and 2 peptides in 4 patients. In long-term survivors, the numbers of peptides, against which increased CTL responses were observed, were 0 peptide in 5 patients, 1 peptide in 12 patients, 2 peptides in 1 patient and 3 peptides in 2 patients ($p = 0.827009$).

Discussion

This study showed that both lymphocyte counts prior to the vaccination and increased IgG response to the vaccinated peptides, along with performance status, well correlated with overall survival of advanced cancer patients who received personalized peptide vaccination. Lymphocyte counts prior to vaccination shall be a biomarker primarily because lymphocytes are absolutely required for vaccine-mediated immune boosting. In addition, lymphopenia is recently reported to be an independent prognostic factor for overall survival in advanced cancers.[3,4] In contrast to lymphocyte counts, one might question why IgG response, but not CTL response, is a biomarker of the effectiveness of the peptide vaccination given that the vaccination primarily activates peptide-specific CTLs, but not B cells. We also brought up the same question when reporting on IgG responses as a biomarker following an investigation of 211 patients under treatment with personalized peptide vaccination.[10] Therefore, we extended that study in the present work and report convincing results showing that IgG response is superior to

Table 1B. Characteristics, Immune responses and clinical responses of 500 patients with advanced cancer

Characteristics	Groups of cancer											
	NSCLC		RCC		Melanoma		Brest cancer		Urotherial cancer		Others	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
No. of patients	22	4	13	3	12	2	11	2	10	2	31	6
Average Age, years	60.5		57.8		57.3		54.3		66.6		63.6	
Standard deviation	12.4		11.2		18.2		11.4		10.7		11.9	
Sex												
Male	11	50	11	85	7	58	0	0	9	90	16	52
Female	11	50	2	15	5	42	11	100	1	10	15	48
Performance status (ECOG)												
0	14	64	10	77	7	58	5	46	6	60	16	52
1	5	23	2	15	3	25	4	36	3	30	8	26
2	3	13	1	8	2	17	1	9	1	10	7	22
3	0	0	0	0			1	9	0	0	0	0
Peptides bind for HLA												
A2	4	18	3	23	4	33	4	36	4	40	8	26
A24	18	82	9	69	8	67	7	64	6	60	16	52
A3-supretype	0	0	0	0	0	0	0	0	0	0	2	6
Mixed type	0	0	1	8	0	0	0	0	0	0	5	16
Average times of vaccination	13.8		23.5		12.3		9		11.9		10.8	
Standard deviation	15.4		15		6.6		9.8		6		13.4	
Treatment												
Vaccination alone	22	100	12	92	12	100	4	36	9	90	29	94
Combination	0	0	1	8	0	0	7	64	1	10	2	6
CTL response												
No. of evaluable case	11		10		8		6		3		12	
yes	6	55	2	20	6	75	1	17	2	67	4	33
no	5	45	8	80	2	25	5	83	1	33	8	67
IgG response												
No. of evaluable case	12		9		7		4		3		16	
yes	7	58	5	56	5	71	4	100	2	67	7	44
no	5	42	4	44	2	29	0	0	1	33	9	56
Best clinical response												
No. of evaluable case	21		12		11		10		7		23	
PR	0	0	0	0	0	0	0	0	1	14	0	0
SD	11	52	9	75	5	45	1	10	2	29	8	35
PD	10	48	3	25	6	55	9	90	4	57	15	65
Response rate (%)									14.3			
Disease control rate (%)	52.4		75		45.5		10		42.9		34.8	

Immunological responses were evaluated using the pre-and post-sixth vaccination samples.

CTL response in predicting the overall survival of advanced cancer patients under treatment with personalized peptide vaccination.

It is obvious that cellular immune responses shall be an important marker if appropriate assay conditions are defined and used. However, the current available T cell assays possess insufficient sensitivity and reproducibility for monitoring immune responses in vaccinated patients. Various T cell assays for quantifying and characterizing antigen-specific T cell responses, including ELISPOT, ELISA, intracellular cytokine staining (ICS), ^{51}Cr-release cytotoxicity assay, peptide-MHC multimer and proliferation assay (^{3}H-thymidine uptake and CFSE), have been extensively studied.[4,30,31] Using these T cell assays, increasing numbers of studies have reported significant correlations between clinical benefits and immunological responses in a limited number of patients.[4,30,31] However they are often inconsistent and unreproducible in other studies, because no universal standards have been established in the current T cell assays, which continue to be modified on a regular basis.[4,30,31] In fact, we have already tried several T cell assays, including delayed type hypersensitivity test and cytotoxicity assay, in our vaccinated patients, but their results were no better than the CTL precursor assay that we employed in the current study.[10] We also employed ELISPOT assay with the similar results (Noguchi M, et al., unpublished results). Therefore, we think that optimization and

standardization of T cell assay protocols, including the analysis, interpretation and reporting of data, may be crucial for future development of immune monitoring in cancer patients.[4,30,31] Nevertheless, it should be also noted that T cell assays have their inherent limitations. Even if innovated technologies are introduced and assay protocols are sophisticated, it will be difficult to dramatically improve their performance characteristics, such as sensitivity and reproducibility, because the frequencies of antigen-specific T cells are usually quite low even after vaccination.[5,6]

One might have several questions with regard to relationship between peptide-specific CTL responses and peptide-specific IgG responses, but we found no statistically significant correlation between the increased IgG responses and the increased CTL responses in 300 patients shown in Table 1A and B as well as 43 patients shown in Table 4A and B. We previously reported that both IgG and CTL responses were augmented in the samples after 6th vaccination from the majority of patients who showed PR responses.[19,23,25] We also demonstrated that there were no significant differences in overall survival between patients showing both CTL and IgG responses and those showing only IgG response.[10,11] These results suggest that boosted CTL responses are involved in tumor reduction, but not necessarily involved in prolonged overall survival.

We investigated the correlation between pre-vaccination lymphocyte counts and the induction of IgG responses in the 43 patients listed in Table 4A and B. As a result, there was no significant correlation between them. In addition, we addressed if boosted IgG responses to the vaccinated peptides were associated with concomitant increase of peptide-specific IgG to non-vaccinated peptides in the patients showing longer survivals shown in Table 4A and B. As a result, no such concomitant increase was observed in the majority of long survivors as well as short survivors listed in Table 4A and B. These results suggest that the boosting effect was really limited to the vaccinated peptides.

There could be several possible explanations for these unexpected results. Firstly, to the best of our knowledge, none of the previously reported studies involved more than several hundred cases under a single concept (personalization of peptide selection) of therapeutic peptide vaccination for advanced cancer patients. Although some of the clinical trials of peptide vaccination identified CTL response as a biomarker that predicts overall survival,[2,4,10,30] the numbers of patients were too small to obtain significant results. Furthermore, the clinical benefits of those peptide vaccination trials were not sufficiently large to enter randomized phase III trials. A number of poorly validated or controversial markers made it difficult to obtain approval of cancer vaccines as drugs. Indeed, there are no prospectively defined markers validated in large phase II or III studies at the time of writing.[7-9] Therefore, IgG response, but not CTL response, to the vaccinated peptides or proteins has the possibility to become a true biomarker that is predictive of the overall survival of cancer patients under treatment with cancer vaccine. In line with our observations, other researchers have also recognized the significance of B cell responses induced by vaccination with tumor antigens. Secondly, we previously reported that the personalized peptide vaccination mainly induced infiltration of CD45RO+ T cells, but not that of CD8+ T cells or CD20+ B cells.[31] The results suggest that personalized peptide vaccination initially induced CD45RO+ memory helper T cells to infiltrate into tumor sites, which in turn facilitated the proliferation of CD8+ CTLs and B cells. Consequently, the activated CTLs eliminated cancer cells, while the activated B cells differentiated into plasma cells, which in turn produced IgG specific to the vaccinated peptides. Although the precise mechanisms, in which helper CD4+ T cells are activated after vaccination with HLA class I-restricted

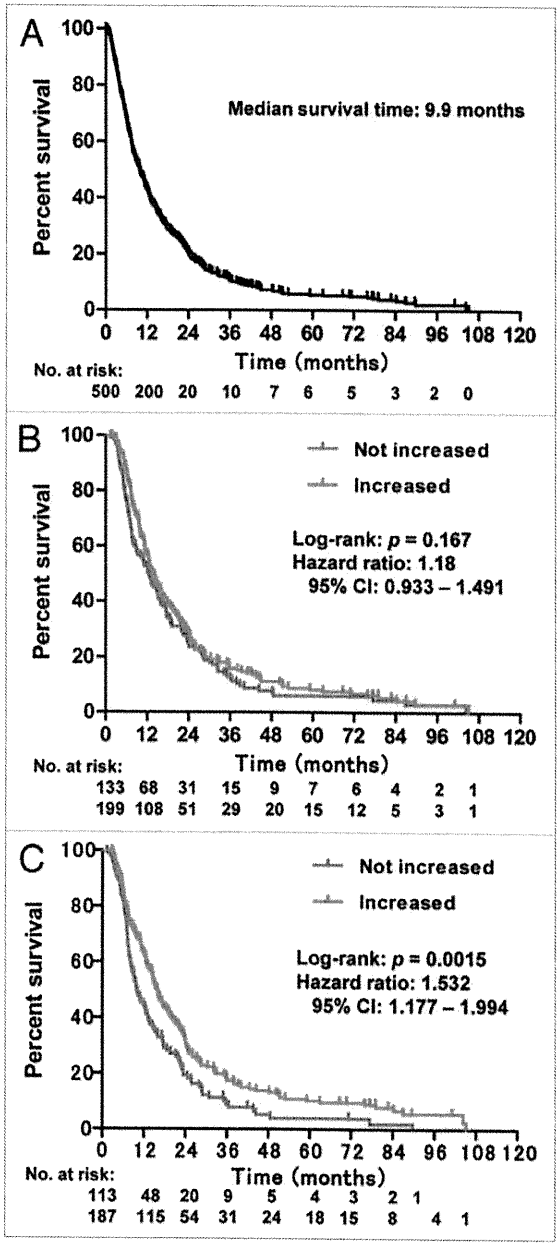

Figure 1. (A) Overall survival in 500 patients with advanced cancer treated by personalized peptide vaccination. Overall survival curves according to peptide-specific cellular (B) and humoral (C) immune response status.

Table 2. Univariate and multivariate analysis for overall survival using Cox regression models

	Univariate			Multivariate		
Factor	**p**	**HR**	**95% CI**	**p**	**HR**	**95% CI**
Performance status (ECOG) ≥ v < 1	<0.0001	2.4560	1.990–3.030	<0.0001	2.2950	1.653–3.188
Counts of lymphocytes < v ≥ 1,500/μL	<0.0001	1.6810	1.362–2.074	0.0095	1.4720	1.099–1.972
IgG responses no v yes	0.0015	1.4970	1.167–1.919	0.0116	1.4550	1.087–1.948
Age < v ≥ 63	0.0020	1.3420	1.113–1.617	-	-	-
Gender Male v Female	0.0984	0.8420	0.686–1.033	-	-	-
CTL responses no v yes	0.1587	1.1800	0.937–1.486	-	-	-
HLA typing A24 v others	0.2504	0.8900	0.729–1.086	-	-	-
Vaccine interval 1 week v ≥ 2 weeks	0.2117	0.8760	0.712–1.078	-	-	-

Lymphocyte and patient age are based on median values, and the remaining are treated as dichotomous variables.

Table 3. Baceline patient characteristics

	Long survivors		Short survivors		
	No	**%**	**No**	**%**	**p**
No. of patients	20		23		
Age, years					
Median	71		64		0.152
Range	54–78		50–80		
ECOG performance status					
0	20	100	20	87	0.236
1			3	13	
HLA typing					
A24	10	50	13	57	0.761
A2	8	40	8	35	
A24 and A2	2	10	2	8	
PSA, ng/ml					
Median	34.5		83		0.404
Range	2–330		2–296		
Gleason score					
7	6	30	3	13	0.299
8	9	45	10	43.5	
9	5	25	10	43.5	
Site of metastasis					
No	3	15	2	9	0.651
Bone only	14	70	17	74	
Bone and node	2	10	3	13	
Node/organ	1	5	1	4	
Progression free survival time, days					
Median	57		43		0.042
Range	14–926		14–96		
Survival time, days					
Median	1483		189		<0.0001
Range	699–2811		79–297		

peptides, still remain to be clarified, one possibility is that the peptides employed in this study may be presented not only in HLA class I but also in HLA class II and recognized by both CD8 and CD4 T cells, as has been reported in the PSA peptide at position 248–257 in prostate patients by our group and also in the Melan A 26–35 (A27L) peptide in melanoma patients.[32,33] Alternatively, the peptides employed in this study may be recognized by $CD4^+$ T cells on HLA class I molecules without requirement of CD8

Table 4A. Comparison of immune responses between short- and long-term survivors

		Short-term survivors (n = 23)					
		Anti-peptide cellular response			Anti-peptide IgG response		
Pts no.	Peptide	Pre	Post (sixth)	Increased response	Pre	Post (sixth)	Increased response
1	SART3-109	0	NT	NA	492	1221	≥2
	Lck-208	0	NT	NA	11	18	negative
	Lck-488	0	NT	NA	15	20	negative
	SART3-315	0	NT	NA	30	27	negative
2	SART3-109	53	183	≥2	456	3123	≥2
	Lck-488	159	0	negative	320	310	negative
	ART1-170	1312	0	negative	<10	<10	negative
	SART3-315	77	189	≥2	<10	<10	negative
3	SART2-161	899	0	negative	36	38	negative
	Lck-208	323	108	negative	<10	<10	negative
	Lck-486	101	0	negative	118	144	negative
	SART3-315	53	69	negative	35	30	negative
4	SART3-109	41	NT	NA	22	14	negative
	Lck-208	67	NT	NA	<10	<10	negative
	Lck-486	78	NT	NA	107	92	negative
	ART4-75	79	NT	NA	NT	NT	NA
5	CypB-172	212	NT	NA	<10	1211	≥10
	HNRL-501	477	NT	NA	<10	18	≥10
	ppMAPkkk-294	0	NT	NA	12	13	negative
6	PAP-213	159	0	negative	34	39	negative
	PSA-248	55	0	negative	273	2138	≥2
	SART3-315	449	0	negative	<10	<10	negative
	PSA-152	516	61	negative	<10	<10	negative
7	UBE-43	0	NT	NA	308	NT	NA
	UBE-208	223	NT	NA	73	NT	NA
	PSCA-21	0	NT	NA	143	NT	NA
	EGFR-479	74	NT	NA	68	NT	NA
8	UBE-43	0	NT	NA	544	NT	NA
	PSCA-21	56	NT	NA	358	NT	NA
	PTHrP-42	0	NT	NA	176	NT	NA
	Her2/neu-484	0	NT	NA	227	NT	NA
9	SART3-302	608	NT	NA	229	19363	≥10
	Lck-422	0	NT	NA	14	215	≥2
	WHSC2-103	0	NT	NA	48	70	negative
	UBE2V-43	0	NT	NA	35	59	negative
10	SART3-109	5561	0	negative	274	283	negative
	Lck-488	0	0	negative	98	96	negative
	MRP3-1293	0	0	negative	78	76	negative
	PAP-213	0	0	negative	68	69	negative

NA, not available; NT, not tested. [a]Values indicate IFNγ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFNγ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFNγ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 10.

Table 4A. Comparison of immune responses between short- and long-term survivors (continued)

11	SART3-309	NT	NT	NA	199	537	≥2
	CypB-129	NT	NT	NA	804	530	negative
	UBE-43	NT	NT	NA	41	28	negative
	HNRL-501	NT	NT	NA	35	26	negative
12	SART2-93	0	0	negative	12	12	negative
	Lck-208	68	0	negative	15	11	negative
	Lck-486	123	348	≥2	21	<10	negative
	CypB-91	0	0	negative	15	11	negative
13	CypB-172	488	1000	≥2	151	<10	negative
	Lck-422	0	0	negative	12	<10	negative
	MAP-294	0	0	negative	41	21	negative
	HNRL-501	0	0	negative	15	16	negative
14	SART3-109	0	2045	≥10	9524	7283	negative
	Lck-208	0	2246	≥10	0	0	negative
	Lck-488	118	184	negative	70	86	negative
	PSA-248	0	0	negative	8	11	negative
15	SART3-109	0	0	negative	561	780	negative
	PAP-213	0	0	negative	112	125	negative
	PSA-248	0	0	negative	251	271	negative
	PSA-152	109	0	negative	29	<10	negative
16	SART3-302	0	931	≥10	251	223	negative
	CypB-172	0	0	negative	312	350	negative
	Lck-246	0	4326	≥10	186	199	negative
	ppMAPkkk-294	0	0	negative	132	126	negative
17	Her2/neu-553	0	NT	NA	31	NT	NA
	EZH2-291	0	NT	NA	26	NT	NA
	PTHrP-102	0	NT	NA	10	15	negative
	PSA-248	0	NT	NA	45	822	≥10
18	PAP-213	0	1289	≥10	534	18980	≥10
	PSA-248	0	0	negative	103	9855	≥10
	Her2/neu553	0	302	≥10	59	89	negative
19	SART3-109	0	0	negative	879	930	negative
	Lck-488	0	0	negative	641	663	negative
	PAP-213	0	191	≥10	143	138	negative
20	SART3-302	0	0	negative	11	10	negative
	UBE2V-43	0	0	negative	40	43	negative
	HNRL-501	0	0	negative	10	12	negative
	EZH2-569	0	130	≥10	38	39	negative
21	SART3-109	0	0	negative	287	7040	≥10
	Lck-486	0	0	negative	232	334	negative
	PAP-213	0	131	≥10	91	21230	≥10
	EZH2-291	753	0	negative	341	14258	≥10

NA, not available; NT, not tested. [a]Values indicate IFNγ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFNγ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFNγ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 10.

Table 4A. Comparison of immune responses between short- and long-term survivors (continued)

22	SART2-161	0	4923	≥10	24	90	≥2
	SART3-109	318	0	negative	141	151	negative
	Lck-486	0	0	negative	39	41	negative
	MRP3-1293	262	0	negative	30	30	negative
23	SART3-109	0	NT	NA	945	7675	≥2
	Lck-486	0	NT	NA	18	18	negative
	MRP3-1293	0	NT	NA	16	16	negative

NA, not available; NT, not tested. [a]Values indicate IFNγ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFNγ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFNγ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 10.

molecules, as has been reported on T cell receptor-engineered $CD4^+$ T cells.[34] Although we have no data on the association between HLA class II types in the vaccinated patients and anti-peptide IgG responses in the current study, this important issue will be addressed in further studies. Biological roles of peptide-specific IgG also need to be elucidated in the near future.

Increases IgG responses to the vaccinated peptides in patients showing longer survival could be, at least in part, in reflection of their better immune-competence with regard to helper T cell functions and subsequent B cell responses, although biomarkers predictable of better immune-competence with regard to favorite clinical benefits in response to peptide vaccinations are presently unclear. This issue is now under investigation and our preliminary results suggest that serum levels of C-reactive protein could be one of them (Noguchi M, et al. unpublished results). At the literature level, a number of prognostic factors have been evaluated with respect to their roles in determining the treatment strategy and ability to predict the response to therapy. Recent reports have shown some significant prognostic factors for CRPC patients. Smaletz et al. reported that performance status, lactate dehydrogenase (LDH), PSA and alkaline phosphate were significant prognostic factors of overall survival in HRPC patients.[35] Halabi et al. reported that performance status, Gleason sum, LDH, alkaline phosphatase, PSA, hemoglobin and visceral metastases were associated with survival in CRPC patients.[36] Unlike these reports, we identified the number of lymphocytes before vaccination and IgG responses after vaccination. These factors were not included in the other reports because most patients in the above studies were treated without specific active immunotherapy.

To address whether or not the long-term survived HRPC patients shown in **Table 4A and B** were different from "better performing, more likely to survive" patients who are not treated with cancer vaccines, we compared the results shown in this study with those of the TAX327 study of docetaxel-based regimens without the vaccine treatment, as a well known historical control, primarily because the disease conditions of HRPC patients in the TAX327 study were similar to those of this study subjects.[13,37] Namely, in the TAX327 study, a randomized, nonblinded, multinational phase III study involving 1,006 men with HRPC, they had a median survival of 16 to 20 months.[13,37] In that study, there were 800 deaths (80%) of 1,006 patients within 18 months of follow-up.[38] Therefore, long-term survivors for more than 30 months (900 days) shown in **Table 4A and B** could be considered to benefit from the peptide vaccination, and thus could be different from better performing HRPC patients who received the standard therapy without cancer vaccines. Of note, the beneficial roles of our personalized peptide vaccination have been also clearly demonstrated in the recently conducted randomized trial in consideration of the pre-existing host immunity.[39] Although several papers[2,4,39] have been reported on the relationships between lymphocyte counts and survival in advanced cancers, there have been no publications regarding antibody responses after peptide vaccinations and survival in cancer patients. Because all of our data were derived from the cancer patients that might have received a survival benefit from vaccinations, we cannot know whether the patients who were able to mount an antibody response and who were not lymphopenic were in fact more likely to control the cancer (and survive longer) even if they did not receive the vaccine. To address this issue, we will need to examine anti-peptide IgG responses after vaccinations with antigen peptides that do not affect patient survival. However, it would be very difficult for us to obtain such data.

One might have a question whether the IgG responses to the vaccinated peptides are unique to the peptides used in this study or widely observed in peptide vaccines conducted in other groups. Unfortunately, to our knowledge, no other groups have examined anti-peptide IgG responses after peptide vaccinations in the literature. Therefore, it would be impossible for us to decide whether the IgG responses that we detected in this study are unique to our peptide vaccines or not. Also, we do not know at the present time whether anti-peptide IgG responses are useful in general as an indicator of survival in cancer patients without vaccinations, because all of our data were derived from the cancer patients that received peptide vaccinations. Of note, however, the methods to identify the peptides used in this study are largely different from those by other groups. We at first established tumor-specific CTL clones and lines in culture of patients' PBMCs and autologous tumor cell lines, followed by identification of genes

Table 4B. Comparison of immune responses between short- and long-term survivors

		Long-term survivors (n = 20)					
		Anti-peptide cellular response			Anti-peptide IgG response		
Pts no.	Peptide	Pre	Post (sixth)	Increased response	Pre	Post (sixth)	Increased response
24	SART2-93	83	0	negative	23	106	≥2
	SART3-109	0	922	≥10	252	11618	≥10
	Lck-488	116	85	negative	120	337	≥2
	PSMA-624	154	0	negative	58	276	≥2
25	Lck-208	71	0	negative	<10	<10	negative
	ART1-170	101	74	negative	<10	35	≥10
	ART4-75	101	0	negative	24	510	≥10
	CypB-84	141	0	negative	<10	<10	negative
26	CypB-129	0	0	negative	43	149	≥2
	Lck-246	0	0	negative	1155	22853	≥10
	HNRL-501	120	729	≥2	<10	51	≥10
	EIF-51	169	0	negative	NT	NT	NA
27	SART3-109	0	0	negative	1107	26809	≥10
	SART3-315	0	0	negative	<10	151	≥10
	Lck-208	60	0	negative	169	142	negative
	PSA-152	106	0	negative	81	114	negative
28	SART3-109	0	700	≥10	57	67	negative
	Lck-488	194	0	negative	90	94	negative
	MRP3-1293	108	0	negative	20	37	negative
	PSMA-624	133	0	negative	55	66	negative
29	SART2-161	143	0	negative	27	15	negative
	SART3-109	0	1032	≥10	263	418	negative
	Lck-488	208	0	negative	81	56	negative
	PSA-248	0	0	negative	70	78	negative
30	UBE2V-43	0	720	≥10	48	5083	≥10
	EIF4EBP-51	261	294	negative	23	83	≥2
	PSA-170	565	0	negative	62	32	negative
	EGF-R-479	0	0	negative	71	67	negative
31	SART3-109	80	3502	≥10	121	115	negative
	Lck-486	0	0	negative	45	35	negative
	SART1-690	0	0	negative	301	426	negative
	SART2-899	677	147	negative	98	260	≥2
32	MAP-432	64	480	≥2	178	1259	≥2
	Lck-246	0	720	≥10	11	451	≥10
	Lck-422	0	130	≥10	13	15	negative
	UBE-43	0	0	negative	15	1534	≥10
33	SART3-309	59	0	negative	142	179	negative
	CypB-172	324	0	negative	129	121	negative
	WHSC-103	70	0	negative	<10	<10	negative

NA, not available; NT, not tested. [a]Values indicate IFN-γ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFN-γ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 10.

Table 4B. Comparison of immune responses between short- and long-term survivors (continued)

	WHSC-141	0	0	negative	<10	<10	negative
34	SART3-315	0	0	negative	NT	NT	NA
	PSA-248	0	0	negative	29	4413	≥10
	PSM-624	0	0	negative	<10	<10	negative
	PAP-213	0	0	negative	41	25783	≥10
35	SART2-93	51	912	≥2	99	934	≥2
	Lck-488	108	61	negative	74	721	≥2
	PSA-152	0	0	negative	10	900	≥10
	PSA-248	0	0	negative	717	1058	negative
36	SART3-109	0	0	negative	184	228	negative
	Lck-208	56	0	negative	23	28	negative
	PAP-213	57	1802	≥10	13	379	≥10
	SART3-315	158	1121	≥2	NT	NT	NA
37	SART3-302	0	1417	≥10	40	11118	≥10
	SART3-309	0	0	negative	108	424	≥2
	PSA-170	0	0	negative	21	1221	≥10
	PSA-178	0	0	negative	32	1889	≥10
38	SART3-302	0	0	negative	309	15523	≥10
	CypB-129	0	0	negative	91	858	≥2
	PSMA-441	0	1163	≥10	NT	NT	NA
	PSMA-711	0	0	negative	NT	NT	NA
39	SART3-109	0	282	≥10	134	9562	≥10
	Lck-486	449	126	negative	14	12	negative
	PSA-248	157	172	negative	12	14507	≥10
	PTHrP-102	209	119	negative	16	11256	≥10
40	SART2-161	81	0	negative	1433	1451	negative
	SART3-109	0	0	negative	5368	24796	≥2
	PSA-248	0	0	negative	47	3854	≥10
	EZH2-291	0	784	≥10	2027	6674	≥2
41	SRAT3-109	0	0	negative	170	992	≥2
	Lck-488	0	0	negative	54	30278	≥10
	MRP3-1293	312	0	negative	21	3996	≥10
	PSA-248	78	0	negative	25	29669	≥10
42	CypB-129	464	0	negative	348	468	negative
	HNRL-501	0	436	≥10	859	1298	negative
	EIF-51	0	102	≥10	714	6797	≥2
	EZH2-569	0	899	≥10	2501	305	negative
43	CypB-129	140	0	negative	26	38	negative
	UBE-43	141	3424	≥10	27	1910	≥10
	EZH2-569	313	417	negative	18	446	≥10
	Her2-484	69	0	negative	<10	15	≥10

NA, not available; NT, not tested. [a]Values indicate IFN-γ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFN-γ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 10.

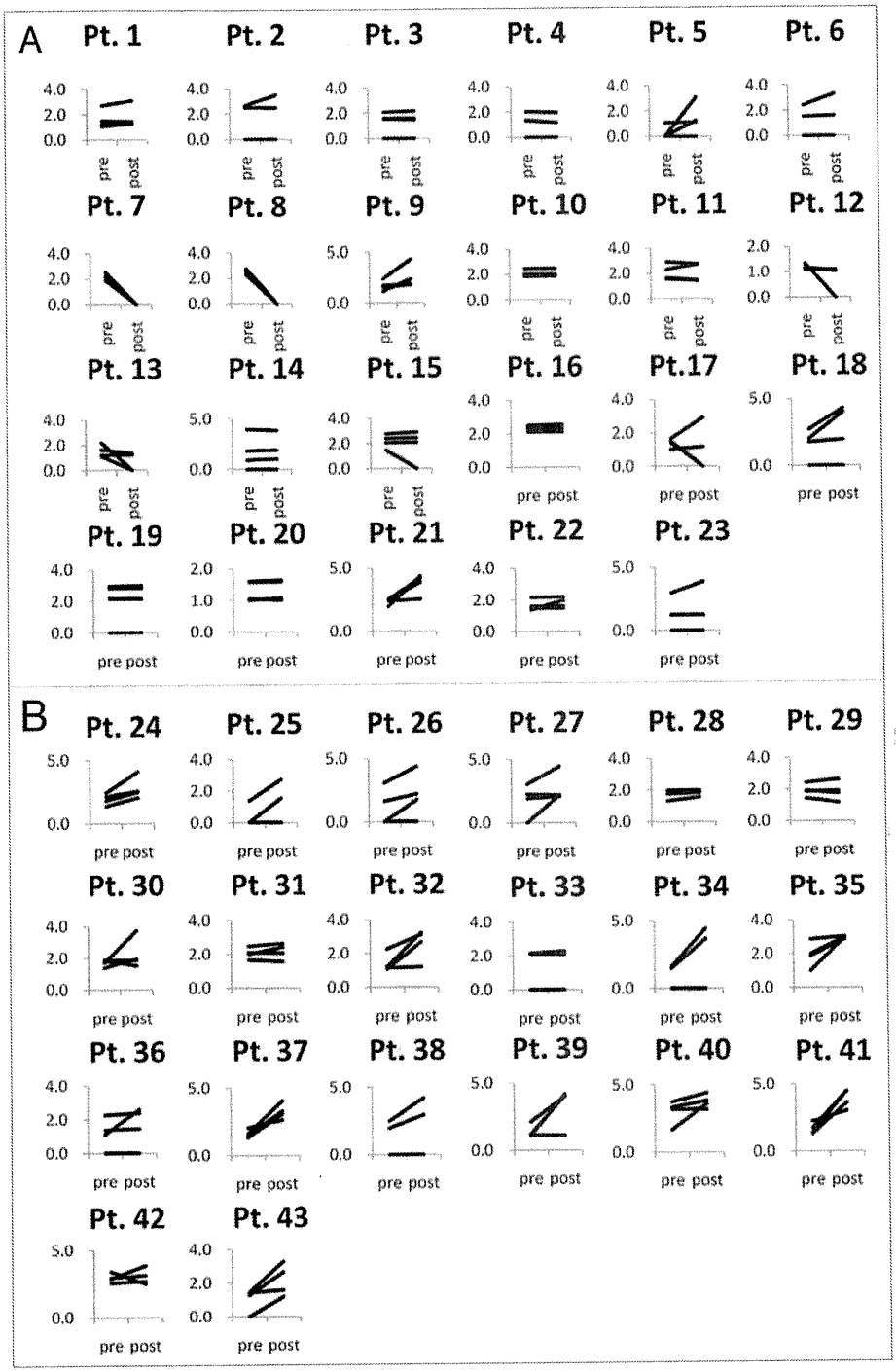

Figure 2. Changes of IgG levels reactive to each of the vaccinated peptides during pre- and post-vaccination periods (sixth) for short-term survivors (A) and long-term survivors (B). The vertical bars denote log 10 scores in order to better represent n-fold increases in IgG levels. NA, not available.

encoding tumor associated antigens by means of cDNA expression cloning technique reported by Boon et al.[40] Among many peptide candidates coded by these antigens, the peptides capable of inducing CTL reactive to tumor cells in HLA-class IA-restricted and peptide-specific manners were screened by incubation of PBMCs from cancer patients. Interestingly, many of these identified peptides were also recognized by pre-vaccination plasma IgG of cancer patients as reported previously.[41] Subsequently, to save limited source of patients' PBMCs, a large numbers of peptide candidates holding the motifs for binding to HLA-class IA molecules were at first tested for their ability to react to pre-vaccination patients' IgG, followed by testing their ability to induce HLA-class IA-restricted and peptide-specific CTL reactive to tumor cells in patients' PBMCs. Therefore, the peptides employed in this study mainly selected by their ability to be recognized by both cellular and humoral immunity. As far as we know, no other clinical trials of peptide-based cancer vaccine provided such peptides; other groups used the peptides capable of inducing only CTL without paying attention to their reactivity to IgG.

In conclusion, we have shown that IgG response is superior to CTL response as an immunological biomarker that is predictive of the overall survival of advanced cancer patients under treatment with personalized peptide vaccination. These results might provide new insights to better understand biomarkers of cancer vaccine for advanced cancer patients. Application of these results for the other types of cancer vaccine using common proteins or common peptides in a non-personalized manner could be worthy to consider.

Patients and Methods

Study population. This study was conducted through the serial collection of blood samples from 500

consecutive patients positive for HLA-A24, -A2 or -A3 supertypes with advanced cancer, who entered into phase I, I/II and II clinical trials for personalized peptide vaccination at 8 institutions (Kurume University Hospital, Kinki University Hospital, Okayama University Hospital, Hokkaido University Hospital, Niigata University Hospital, Kitasato University Hospital, Kansai Medical University Hospital and Yamaguchi University Hospital, Japan) between October 2000 and October 2008. The ethics review committee of each institution accepted the present project and blood samples were collected at baseline (before vaccination), at sixth vaccination, and during the follow-up period after written informed consent was obtained. All 500 patients suffered from advanced cancer originating in the prostate (n = 174), colon and rectum (n = 74), pancreas (n = 50), stomach (n = 42), brain (n = 33), uterus (n = 28), lung (n = 22), kidney (n = 13), skin (n = 12), breast (n = 11), bladder and urinary tracts (n = 10) and elsewhere (n = 31) (**Table 1A and B**). The safety, immune responses and clinical responses in most of those studied had been reported previously.[6,13-29] The exceptions were the results of vaccinations against bladder cancer, breast cancer, some pancreatic cancer cases, and those from HLA-A3 supertype-positive patients. These unpublished results have now been submitted for publication or are under preparation based on results obtained after October 2008. In the sub-analysis, 20 patients who survived more than 900 days (long-term survivors) and 23 patients who died within 300 days (short-term survivors) were selected to compare immune responses from a total of 174 patients with CRPC.

Personalized peptide vaccination and immunological assessment. Personalized peptide vaccination is based on a pre-vaccination measurement of peptide-specific CTL precursors and anti-peptide IgG in the circulation of cancer patients reactive to vaccine candidates, followed by administration of only reactive peptides (up to four peptides) as reported previously.[25-29] Selected peptides were mixed with incomplete Freund's adjuvant (Montanide ISA-51VG; Seppic, Paris, France), and four peptides of 1.5 ml emulsion each at doses of 3 mg/peptide were injected subcutaneously into the regional lymph node area. A total of 77 candidate peptides (32 peptides for HLA-A24-positive cancer patients, 37 for HLA-A2 and 8 for HLA-A3 supertypes) were used in the personalized peptide vaccination. All of these peptides can induce HLA-A24-, A2- and A3-supertype-restricted and tumor-specific CTL activity in PBMCs of cancer patients.[6,13-29,42-44]

Before the first vaccination and 7 days after every sixth vaccination, 30 ml of peripheral blood was obtained and PBMCs were isolated by means of Ficoll-Conray density gradient centrifugation. Peptide-specific CTL precursors in PBMCs were detected using the previously reported culture method.[25-29] Briefly, PBMCs (1 x 10^5 cells/well) were incubated with 10 μM of a peptide in 200 μl of culture medium in u-bottom 96-well microculture plates (Nunc, Roskilde, Denmark). Half of the medium was removed and replaced with a fresh medium containing a corresponding peptide (20 μM) every 3 days. After incubation for 14 days, these cells were harvested and tested for their ability to produce IFNγ in response to CIR-A2402 or T2 cells that were pre-loaded with either a corresponding peptide or HIV peptides (RYL RQQ LLG I for HLA-A24 and LLF GYP VYV for HLA-A2) as a negative control. For HLA-A3 supertype-positive cases, the cells were harvested and tested for their ability to produce IFNγ in response to CIR-A1101, -A31012 or -A3303 cells that were pre-loaded with either a corresponding peptide or an HIV peptide (RLR DLL LIV TR) as a negative control. The level of IFNγ was determined by enzyme-linked immunosorbent assay (ELISA) (limit of sensitivity: 10 pg/ml). All assays were performed in quadruplicate. A two-tailed Student's t-test was employed for the statistical analyses.

The levels of anti-peptide IgG were measured using the Luminex™ system, as previously reported.[25-29,45] In brief, plasma was incubated with 25 μl of peptide-coupled color-coded beads for 2 h at room temperature on a plate shaker. After incubation, the mixture was washed with a vacuum manifold apparatus and incubated with 100 μl of biotinylated goat anti-human IgG (chain-specific) for 1 h at room temperature. The plate was then washed, followed by the addition of 100 μl of streptavidin-PE to wells and was incubated for 30 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 μl of Tween-PBS to each well. Fifty microliters of sample was used for detection with the Luminex™ system.

For evaluation of immune responses during the treatment, peptide-specific CTL precursors among PBMCs and serum levels of peptide-specific antibodies were measured every sixth vaccination. Positive immune responses were defined as either post (sixth vaccination) IgG levels/pre-IgG levels ≥ 2 or post (sixth vaccination) IFNγ levels/pre-IFNγ levels ≥ 2. In addition, in the analysis between long- and short-term survivors, positive immune responses were defined as either post (sixth vaccination) IgG levels/pre-IgG levels ≥ 10 or post (sixth vaccination) IFNγ levels/pre-IFNγ levels ≥ 10.

Adverse events and clinical responses. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. The clinical responses were evaluated on the basis of clinical observations and radiological findings. Patients were assigned a response category according to the Response Evaluation Criteria in Solid Tumors (RECIST).

Statistical methods. Overall survival and 1 and 3 year survival rates were determined by Kaplan-Meier actuarial analysis and the difference between survival curves was assessed by the log-rank test. Cox proportional hazards regression model was used for univariate and multivariate analyses to identify combinations of factors that had a significant impact on survival. All baseline parameters in the survival and proportional hazards regression analysis were analyzed as dichotomous variables using the overall mean values as cut-off levels. All statistical calculations were carried out using the StatView® program (SAS Institute Inc., Cary, NC). A two-sided significance level of 5% was considered statistically significant.

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Disclosure Statement

Although all authors completed the disclosure declaration, the following authors indicated a financial or other interest that is relevant to the subject matter under consideration in this article.

Employment or Leadership Position

Akira Yamada is a part-time executive of Green Peptide Co.; Consultant or Advisory Role: Kyogo Itoh, Green Peptide Co.; Stock Ownership: Kyogo Itoh, Akira Yamada, Green Peptide Co.; Honoraria: none; Research Funding: Kyogo Itoh, Akira Yamada, Green Peptide Co.; Expert Testimony: none; Other Remuneration: none.

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A Phase I Study of Personalized Peptide Vaccination Using 14 Kinds of Vaccine in Combination With Low-Dose Estramustine in HLA-A24-Positive Patients With Castration-Resistant Prostate Cancer

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BACKGROUND. To evaluate the safety, tolerability, immune response, and antitumor activity of a combination of personalized peptide vaccination (PPV) and estramustine phosphate (EMP) in patients with castration-resistant prostate cancer (CRPC).

METHODS. In a phase I dose-escalation study, four peptides showing the highest levels of peptide-specific immunoglobulin G (IgG) to 14 vaccine candidates (ITK-1) were subcutaneously injected every week in three different dose settings (1, 3, and 5 mg per peptide) for 6 weeks with a low dose of EMP, and the patients were followed by maximum 2 years extension study either weekly or bi-weekly six times PPV as one course with a low dose of EMP.

RESULTS. Fifteen patients were enrolled in the phase I study. No serious treatment-related adverse events were observed. The most common adverse events were grade 2 skin reactions at the injection sites. The maximum acceptable dose of ITK-1 was 8.643 mg. There were no treatment-related systemic adverse events of grade 3 or more, and maximum tolerated dose could not be determined. Cytotoxic T lymphocyte responses measured by interferon-γ release assay were boosted in 10 of 15 (67%) patients, and IgG responses were boosted in 7 of 15 (47%) patients. Twelve patients proceeded to the extension study, and the median survival time was 23.8 months during a median follow-up of 23.8 months.

CONCLUSIONS. PPV treatment for HLA-A24 positive patients with CRPC could be recommended for further stages of clinical trials because of its safety and the higher frequency of boosting immune responses. *Prostate 71: 470–479, 2011.* © 2010 Wiley-Liss, Inc.

KEY WORDS: **personalized peptide vaccine; immunotherapy; phase I study; estramustine phosphate**

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INTRODUCTION

In the initial trials, peptide-based vaccine treatment of cancer patients rarely induced clinical responses and the levels of immune responses was low, indicating that the classical type of peptide vaccines did not have a promising future in the treatment of advanced cancer [1,2]. However, there have been slow but substantial advances in peptide vaccines and dendritic cell (DC)-based vaccines with regard to both clinical responses and immunological markers [3–12].

We previously reported that repeated multiple peptide vaccine regimen planned according to the pre-existing immunity (personalized peptide vaccine: PPV) could prolong the overall survival of patients with advanced cancer, and IgG specific to each peptide can frequently be detected in pre- and post-vaccination plasma [13]. In the previous trial, PPV was administered in 113 patients with advanced cancer, and the levels of peptide-specific cytotoxic T lymphocyte (CTL) precursors were measured by the interferon (IFN)-γ release assay and those of anti-peptide immunoglobulin (IgG) were estimated by enzyme-linked immunosorbent assay (ELISA). The level of anti-peptide IgG was a laboratory marker that predicted clinical responses to the PPV with a positive relationship to overall survival. Further, we showed that 58 patients with castration-resistant prostate cancer (CRPC) treated with a combination therapy of PPV and a low dose of estramustine phosphate (EMP) survived for a relatively long period of 17 months, which was comparable with the results of chemotherapy with docetaxel, and serious adverse events occurred less frequently in the study [4].

ITK-1 is a peptide set consisting of 14 kinds of peptide discovered as a HLA class I epitope, which being developed by Green Peptide Co., Ltd. All the 14 peptide candidates can induce CTLs, and each of them can induce HLA-A24-restricted and tumor-specific CTL activity in peripheral blood mononuclear cells (PBMCs) of cancer patients [14–18]. We have conducted a phase I study on PPV and low-dose EMP in HLA-A24-positive patients with CRPC in order to define the safety, tolerability, and immune and prostate-specific antigen (PSA) responses of this drug combination.

PATIENTS AND METHODS

Patients

This was a multi-center study and approved by each institutional review board (IRB) that evaluated it from the viewpoint of the science and ethics in all four hospitals in Japan before the initiation of the study. Patients who had a histological diagnosis of prostate adenocarcinoma (PC) and progressive disease (PD) by diagnostic imaging (computerized tomography; CT, magnetic resonance imaging; MRI or bone scintigraphy) or PSA after both androgen deprivation therapy either by castration or with luteinizing hormone-releasing hormone (LHRH) agonists and anti-androgen therapy, as well as oral EMP treatment were eligible. PSA progression was defined as at least three consecutive rises in serum PSA taken over 2 weeks apart, in the setting of castration levels of testosterone. Patients were required a washout period of at least 4 weeks before the first vaccination after the completion of prior hormone therapy, hormone-chemotherapy, chemotherapy, or immune therapy. Anti-androgen therapy was discontinued for at least 4 weeks before the first vaccination for patients receiving flutamide and 6 weeks for those receiving bicalutamide. All patients had an Eastern Cooperative Oncology Group performance status of 0 or 1, HLA-A24-positive type, and serum testosterone level $\leq$50 ng/dl, and were maintained on LHRH agonist therapy or castration. Adequate organ functions were required and were defined as white blood cell count $\geq$3,000/mm^3, lymphocyte count $\geq$1,200/mm^3, hemoglobin $\geq$9 g/dl, platelets $\geq$100,000/mm^3, total bilirubin $\leq$1.5 mg/dl, AST and ALT $\leq$2$\times$ (upper normal limit), and serum creatinine $\leq$1.4 mg/dl. Patients with comorbidities including serious cardiovascular, hepatic, nephritic, and hematological diseases $\geq$grade 3 of Common Terminology Criteria for Adverse Events (CTCAE), serious gastric ulcers, and infectious diseases with antibiotic treatment, were excluded. Radiation therapy or immunosuppressive treatment using a systematic steroid within the last 1 year was not permitted. All patients gave written informed consent approved by each IRB.

Study Design

This was a phase I open-labeled dose-escalation study. After a pre-vaccination measurement of peptide-specific IgG in the plasma of patients reactive to 14 kinds of vaccine candidate peptides (ITK-1) with the ability to induce CTLs, patients were treated with 6 weekly subcutaneous administration of the top four peptides showing the strongest antibody responses at three different dose settings (1, 3, and 5 mg/peptide), with daily oral EMP 313.4 mg in the phase I study. This was followed by a maximum of 2 years in an extension study of six PPVs either weekly or bi-weekly as one course. All patients were treated at the hospital during the first 1 week followed by outpatient clinic visits. ITK-1 consists of 14 kinds of peptides: $SART2_{93-101}$, $SART3_{109-118}$, $Lck_{208-216}$, $PAP_{213-221}$, $PSA_{248-257}$, $EGFR_{800-809}$, $MRP3_{503-511}$, $MRP3_{1293-1302}$, $SART2_{161-169}$,

$Lck_{486-494}$, $Lck_{488-497}$, $PSMA_{624-632}$, $EZH2_{735-743}$, and $PTHrP_{102-111}$. All peptides were prepared under Good Manufacturing Practice (GMP) compliance by American Peptide Company (San Diego, CA) and by PolyPeptide Laboratories (San Diego, CA), and were supplied in lyophilized vials; 4 mg, including inactive ingredients, under GMP compliance. Selected peptides were dissolved in 1 ml distilled water and emulsified with 1 ml of incomplete Freund's adjuvant (Montanide ISA-51VG; Seppic, Paris, France), under GMP compliance. Each of four peptides in 0.5 ml emulsion at a dose level of 1 mg/peptide (4 mg/2 ml), 1.5 ml emulsion at a dose level of 3 mg/peptide, and 2.5 mL emulsion at a dose level of 5 mg/peptide were injected subcutaneously into the thigh, the hip or the lower part of trunk area. Each peptide was independently injected nearby. EMP was administered orally as a 156.7 mg capsule, one capsule twice daily, for a total daily dose of 313.4 mg, half of the standard dose of EMP (626.8 mg/day) to avoid immunosuppression as reported in our previous study [19]. From the starting dose of 1 mg/peptide, subsequent dose levels were increased after the evaluation of the safety data by the Data and Safety Monitoring Committee (DSMC) according to the dose escalation design of the protocol. The initial cohort included six patients. If the DSMC recommended proceeding to the next level as a result of the safety evaluation of the prior level, new six patients were enrolled. The highest dose level enrolled three patients at first and was evaluated the safety data by the DSMC to include additional three patients. The maximum acceptable dose (MAD) was defined as the lowest dose level at which at least two-thirds of patients experienced grade 2 or greater injection site reactions after the sixth treatment. The maximum tolerated dose (MTD) was defined as the lowest dose level at which more than one-third of patients experienced grade 3 or greater systemic adverse events caused by ITK-1 after the sixth treatment. Adverse events were graded according to the CTCAE version 3.0 and were coded using MedDRA/J (Medical Dictionary for Regulatory Activities Terminology/Japanese) version 12.0. Patients who experienced no significant (≥CTCAE grade3) adverse events and no disease progression, and signed informed consent were eligible to extend treatment until disease progression or unacceptable adverse events occurred, or the patient met other withdrawal criteria.

Pretreatment and Follow-Up Studies

A complete history, physical examination, and routine laboratory studies, including complete blood counts, biochemical tests, ECG, relevant radiologic studies, PSA, and urinalysis were performed before treatment and repeated after every six injections.

Immune Responses

For evaluation of immune responses, peptide-specific CTL precursors in PBMCs and peptide-specific IgG levels in plasma were measured as described previously [13]. Also, peptide-specific IgG levels were measured using patient's plasma of the screening examination to select the best peptides. Briefly, 30 ml of peripheral blood samples were obtained from each patient to measure peptide specific CTL and IgG prior to vaccination, at the fourth and after the sixth vaccinations, and after every sixth vaccination in the extension study, and then the PBMCs and plasma were isolated by Ficoll–Conray density gradient centrifugation. We reported that the IgG specific to each peptide measured by Luminex system as the fluorescence intensity unit (FIU) could frequently be detected in pre- and post-vaccination plasma, and the level of peptide-specific IgG is a laboratory marker that predicts clinical responses to the PPV with a good relationship to overall survival [13,20]. Therefore, peptides were chosen on the basis of evaluation of peptide-specific IgG levels in plasma. Peptide-specific CTL precursors in PBMCs were detected using a previously reported culture method [21]. Briefly, PBMCs (1×10^5 cells/well) were incubated with 10 μM of each peptide in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark) in 200 μl of culture medium. The culture medium consisted of 45% RPMI-1640 medium, 45% AIM-V® medium (Invitrogen Corp., Carlsbad, CA), 10% FCS, 20 U/ml of interleukin-2 (IL-2), and 0.1 mM MEM nonessential amino acid solution (Invitrogen Corp.), 36 mg/L gentamicin sulfate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) every 3 days for up to 12 days. On the 12th day of the culture, 24 hr after the last stimulation, these cells were harvested, washed three times, and then tested for their ability to produce IFN-γ in response to C1R-A2402 cells preloaded with either a corresponding peptide or HIV peptide (RYLRQQLLGI) as a negative control in HLA-A24. The target cells (C1R-A2402, 1×10^4/well) were pulsed with each peptide (10 μM) for 2 hr, and then effector cells (1×10^5/well) were added to each well with a final volume of 200 μl. After incubation for 18 hr, the supernatants (100 μl) were collected, and the amounts of IFN-γ were measured using an ELISA (limit of sensitivity: 10 pg/ml). All experiments were performed in quadruplicate assay.

Definition of Treatment Outcomes

Outcomes were assessed by post-therapy changes in serum PSA and immune responses. A post-therapy

TABLE I. Baseline Demographics

Characteristics	No. of patients (%)
No. of patients	15
Age, years	
Median	73
Range	63–78
ECOG PS	
0	14 (93)
1	1 (7)
Gleason score	
7	3 (20)
8	5 (33)
9	4 (27)
10	1 (7)
Unknown	2 (13)
PSA (ng/mL)	
Median	39.6
Range	0.2–354.4
Site(s) of metastasis	
None	4 (27)
Lymph node	2 (13)
Bone	6 (40)
Lymph node + bone	1 (7)
Other	2 (13)
Local therapy	
Prostatectomy	4 (27)
EBRT	3 (20)
No definitive local therapy	8 (53)
Hormone therapy	
Primary therapy only	1 (7)
≥2 therapies	14 (93)
Chemotherapy	
EMP	15 (100)
Other	2 (13)

ECOG PS, Eastern Cooperative Oncology Group performance status; PSA, prostate-specific antigen; EBRT, external-beam radiation therapy; EMP, estramustine phosphate.

decrease of PSA to a normal range was defined as a complete response (CR) and a decrease in PSA of ≥50% from baseline was defined as a partial response (PR) in the phase I study. Also, a post-therapy PSA decrease of <50% or an increase >25% from baseline were interpreted as no change (NC) [22] and PSA above 125% of the baseline PSA value was defined as PD. Positive immune responses were defined as post-IgG levels/pre-IgG levels ≥3, post-IFN-γ levels/pre- IFN-γ levels ≥3, respectively. All patients were followed up every 3 months for life. Data, except the survival data, were analyzed by November 2009 using SAS (Statistical Analysis System) software version 9.1.3. The Student's *t*-test and the chi-square test were used to compare quantitative and categorical variables, respectively. Overall survival was calculated from the study registration date to the date of the last follow-up or the death from any cause. The Kaplan–Meier method was used to estimate product-limit estimate curves with the survival data obtained in March 2010. Tests results were considered significant at a two-sided significance level of 5%. The analysis was performed by intent to treat.

RESULTS

Patient Characteristics

Fifteen patients were recruited to the study between April 2006 and September 2007. Patient characteristics are listed in Table I. All patients were HLA-A24-positive, and had hormone and EMP refractory prostate cancer. In addition, all 15 patients were evaluated for the safety and the efficacy of the PPV treatment.

Dose Escalation

The dose-escalation scheme is presented in Table II. Maximum dose escalation preplanned for each peptide of 5 mg/2.5 mL (4 peptides, 20 mg/10 mL) was achieved. There were no treatment-related grade 3 or 4 adverse events or deaths in this study. Grade 2 injection site reactions were observed in two of six patients in the first dose level of 1 mg/peptide, and five of six patients in the second dose level of 3 mg/peptide after the sixth treatment. At the 5 mg/peptide dose

TABLE II. The Results of Dose-Escalation in Phase I Study

Peptides dose level (mg/peptide)	No. of patients		No. of patients	
	Enroll	Discontinued or skipped[a]	MAD (≥grade 2 injection site reaction)	MTD (≥grade 3 systemic treatment-related AE)
1	6	0/6	2/6	0/6
3	6	0/6	5/6	0/6
5	3	3/3	3/3	0/3
Total	15	3/15	10/15	0/15

MAD, maximum acceptable dose; MTD, maximum tolerated dose; AE, adverse event.
[a]Patients were discontinued or skipped the treatment because both widespread grade 2 injection site reactions and patients' own requests.

level, three patients were treated, but the vaccination was skipped or discontinued in all three patients considering the ethical viewpoint because of patients' own requests and physical burden, caused by widespread grade 2 injection site reactions. After these treatment-related adverse events, two of three 5 mg/peptide dose level patients were entered in the extension study and then the dose level was reduced to 3 mg/peptide during treatment. The DSMC reviewed the results and recommended stopping the additional three enrollments for the dose level of 5 mg/peptide. Subsequently, the MAD for PPV was calculated to be 8.643 mg/4 peptide (2.161 mg/peptide) based on the logistic regression model.

Adverse Events

There were no treatment-related serious adverse events and no grade 3 or greater adverse events in the phase I study. In contrast, a grade 3 injection site reaction and a grade 3 pyrexia occurred in one patient each during the extension study. All treatment-related adverse events observed in whole study (phase I and extension study) are listed in Table III. The primary nonhematologic treatment-related adverse events were injection site reaction (93.3%), malaise (33.3%), edema peripheral (33.3%), and fatigue (20.0%). These adverse events were manageable with routine intervention. Hematologic adverse events were, grade 1 white blood cell count increased and grade 1–2 lymphocyte count decreased occurred in 4 of 15 (26.7%) and 3 of 15 (20.0%) patients, respectively. One patient at a dose level of 5 mg/peptide had a grade 1 blood fibrinogen increased, and another patient at a dose level of 3 mg/peptide had grade 1 blood triglycerides increased during the first course, and these changes returned to normal levels on the next course.

Immune Response

The best peptides for each patient were selected based on peptide-specific IgG levels for each peptide at the screening examination (data not shown). The results of the immune response in the first course are given in Table IV. After the sixth vaccination, IgG responses were increased in one of six patients with 1 mg/peptide, four of six patients with 3 mg/peptide, and two of three patients with 5 mg/peptide tested. CTL responses measured by IFN-γ release assay were increased in four of six patients with 1 mg/peptide, six of six patients with 3 mg/peptide, and zero of three patients with 5 mg/peptide tested.

Clinical Response

PSA response after the sixth vaccination was CR in one patient (6.7%) receiving 3 mg/peptide, PR in one patient (6.7%) receiving 1 mg/peptide, and PD in two patients (13.3%) receiving 5 mg/peptide. At the time of data analysis, nine patients had died and all deaths were attributed to prostate cancer or metastases. The median follow-up time for all patients was 23.8 months, ranging from 3.0 to 38.3 months. None of the patients was lost to follow-up during this analysis. The median overall survival was 23.8 months for all 15 patients (95% CI, lower limit was 15.6 months, upper limit was not estimated; Fig. 1).

DISCUSSION

We performed a multicenter, open-label, phase I trial to evaluate the safety, tolerability, immune response, and PSA response of a combination of escalating doses of PPV and low-dose EMP. All patients had hormone and EMP-refractory prostate cancer. The treatment regime was well tolerated at all dose levels, except the injection site reaction at the highest dose level of 5 mg/peptide observed in all three patients enrolled, and no MTD was established in this trial. The most common adverse event was injection site reaction. The concept of dose escalation in a phase I trial to identify an MTD may not be applicable to most therapeutic cancer vaccines [23]. Peptide vaccines based on non-mutated melanoma antigens such as MART-1/Melan A and gp100 were initially evaluated in a phase I setting, at doses ranging from 0.1 to 10 mg [24,25]. However, no toxicity was observed even at the highest doses, and in vitro analysis did not reveal any correlation between the peptide dose and the generation of specific T-cell reactivity from the PBMCs of the vaccinated patients. Neither the safety nor efficacy of the vaccine can be assessed in patients with a blunted immune response since both safety and efficacy depend on the immune response. In contrast, our initial trial for colorectal cancer patients with 0.3, 1, and 3 mg/injections of SART3 peptide showed that a dose of 3 mg/injection was better than that of 0.3 and 1 mg/injection based on the induction of cellular immune responses to both tumor cells and peptides [26]. The current phase I study also showed that a dose of 3 mg/injection was better than those of 1 and 5 mg/injection based on the induction of cellular immune responses to peptides, although total doses of four peptides were 4 mg/2 mL, 12 mg/6 mL, and 20 mg/10 mL. Under these conditions, there were no serious adverse events caused by ITK-1; however, grade 2 injection site reactions were observed in two of six patients receiving 1 mg/0.5 mL/peptide, five of six patients receiving 3 mg/1.5 mL/peptide, and three of three patients receiving 5 mg/2.5 mL/peptide in the phase I study. The vaccination was skipped or discontinued in three of three patients receiving 5 mg/2.5 mL/peptide

TABLE III. Treatment-Related Adverse Events for Castration-Resistant Prostate Cancer

	No. of patients experienced treatment-related adverse events during phase I study/whole study[a] by grade									Total (15 patients)	
	1 mg/peptide group (6 patients)			3 mg/peptide group (6 patients)			5 mg/peptide group (3 patients)			All grade	
MedDRA/J ver12.0 symptom: preferred Trem(PT)	G1 (PI/ Whole)	G2 (PI/ Whole)	G3 (PI/ Whole)	G1 (PI/ Whole)	G2 (PI/ Whole)	G3 (PI/ Whole)	G1 (PI/ Whole)	G2 (PI/ Whole)	G3 (PI/ Whole)	P I	Whole
Vomiting	1/1									1 (6.7%)	1 (6.7%)
Ventricular extrasystoles	0/1										1 (6.7%)
Fatigue	0/1	0/1		1/0	0/1					1 (6.7%)	3 (20.0%)
Injection site reaction	2/2	2/3		1/1	5/4	0/1		3/3		13 (86.7%)	14 (93.3%)
Malaise	1/2			0/1	0/1		0/1			1 (6.7%)	5 (33.3%)
Oedema peripheral	1/2	0/1			0/1		0/1			1 (6.7%)	5 (33.3%)
Pyrexia						0/1					1 (6.7%)
Aspartate aminotransferase increased	0/1										1 (6.7%)
Blood fibrinogen increased							1/1			1 (6.7%)	1 (6.7%)
Blood triglycerides increased				1/1						1 (6.7%)	1 (6.7%)
Crystal urine present	0/1										1 (6.7%)
Blood urine present				0/1							1 (6.7%)
Lymphocyte count decreased	1/1	1/1			1/1					3 (20.0%)	3 (20.0%)
Neutrophil count increased	0/1										1 (6.7%)
Urinary casts	0/1										1 (6.7%)
White blood cell count increased	0/1			1/2			1/1			2 (13.3%)	4 (26.7%)
White blood cells urine positive	0/1			0/1							2 (13.3%)
Bacteria urine identified				0/1							1 (6.7%)
Dizziness				0/1							1 (6.7%)
Dizziness postural				0/1							1 (6.7%)
Headache				1/0	0/1					1 (6.7%)	1 (6.7%)
Insomnia		0/1									1 (6.7%)
Cough	0/1										1 (6.7%)
Rash generalized					0/1						1 (6.7%)

[a]Whole study means phase I and extension study.

TABLE IV. Immunorogical Responses During the Personalized Peptide Vaccination

			Anti-peptide IgG response (FIU)[a]				Anti-peptide cellular response (pg/ml)[b]			
Dose of peptide	Pts No.	Peptide	Pre	Post (fourth)	Post (after sixth)	Increased response (after sixth)	Pre	Post (fourth)	Post (after sixth)	Increased response (after sixth)
1 mg	1	Lck-486	94	90	81	—	ND	ND	ND	—
		PSMA-624	<5	<5	<5	—	ND	ND	ND	—
		PTHrP-102	42	30	23	—	113	ND	ND	—
		SART3-109	31	24	21	—	ND	ND	ND	—
	2	Lck-486	310	206	976	Positive	667	ND	204	—
		MRP3-1293	38	21	28	—	ND	ND	186	Positive
		SART2-93	20	11	9	—	ND	ND	656	Positive
		SART3-109	27	13	18	—	899	ND	ND	—
	3	Lck-486	102	102	114	—	ND	78	ND	—
		Lck-488	45	46	52	—	462	ND	ND	—
		MRP3-1293	52	45	50	—	ND	ND	ND	—
		PAP-213	252	210	215	—	ND	ND	ND	—
	4	Lck-486	200	199	247	—	ND	ND	1,393	Positive
		Lck-488	<5	<5	<5	—	ND	ND	472	Positive
		PSA-248	117	99	109	—	ND	ND	ND	—
		PTHrP-102	171	138	142	—	564	ND	ND	—
	5	Lck-486	575	364	396	—	ND	117	57	—
		Lck-488	144	102	92	—	ND	ND	439	Positive
		MRP3-1293	91	64	51	—	133	160	ND	—
		PAP-213	90	70	77	—	3,764	ND	114	—
	6	MRP3-1293	779	586	411	—	ND	477	ND	—
		PSA-248	804	756	1,825	—	ND	ND	ND	—
		PTHrP-102	502	414	310	—	ND	93	753	Positive
		SART3-109	142	152	83	—	ND	ND	3,276	Positive
3 mg	7	Lck-486	202	216	9,028	Positive	ND	1,636	ND	—
		MRP3-1293	29	21	22	—	ND	ND	ND	—
		PAP-213	<5	<5	5	—	274	ND	1,494	Positive
		PSA-248	11	12	1,902	Positive	173	ND	ND	—
	8	Lck-486	298	261	287	—	2,543	ND	ND	—
		Lck-488	10	9	11	—	ND	ND	598	Positive
		MRP3-1293	23	21	23	—	ND	ND	ND	—
		PAP-213	8	5	9	—	ND	ND	2,613	Positive
	9	Lck-486	329	290	308	—	ND	ND	72	—
		Lck-488	128	103	106	—	ND	119	627	Positive
		MRP3-1293	53	36	40	—	ND	1,706	ND	—
		PAP-213	<5	<5	10,992	Positive	ND	683	ND	—

(*Continued*)