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Phase II study of personalized peptide vaccination for refractory bone and soft tissue sarcoma patients

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Refractory bone and soft tissue sarcomas are challenging diseases to treat because of their robustness to chemotherapy. Although cancer vaccines have the potential to become an attractive treatment modality, their progress has been hampered by the presence of many subtypes of sarcomas and different human leukocyte antigen (HLA)-types. We investigated whether personalized peptide vaccination (PPV) would be feasible for the vast majority of sarcoma patients. Twenty refractory bone and soft tissue sarcoma patients with nine different subtypes and 11 different HLA-class IA phenotypes were enrolled in this study. A maximum of four HLA-matched peptides showing higher peptide-specific IgG responses in pre-vaccination plasma were selected from 31 pooled peptide candidates applicable for the HLA-A2, -A3, -A11, -A24, -A26, -A31, and -A33 types, and were subcutaneously administered weekly for 6 weeks and bi-weekly thereafter. Measurement of peptide-specific CTL and IgG responses along with other laboratory analyses were conducted before and after vaccination. No patients were excluded by either sarcoma subtypes or different HLA-types. No severe adverse events associated with PPV were observed in any patients. Peptide-specific immunological boosting was observed in the post-vaccination samples from the majority of patients. Tumor reduction of the lung metastasis and a long stable disease was observed in each case, and the median overall survival time of the 20 cases was 9.6 months. Taken together, PPV could be feasible for the vast majority of refractory sarcoma patients because of the safety and higher rates of immunological responses regardless of the presence of different sarcoma subtypes and various HLA-types. (*Cancer Sci* 2013; 104: 1285–1294)

Refractory bone and soft tissue sarcomas are challenging diseases to treat with an unmet need for effective systemic therapy.[1,2] Several molecularly targeted agents, such as mammalian target of rapamycin (mTOR) inhibitor[3] and antibody to the insulin-like growth factor 1 receptor (IGF-1R),[4] have shown clinical benefits in a subgroup of sarcoma patients with refractory sarcomas, achieving a median survival time (MST) of 7.6–9.2 months. However, new treatment modalities still remain to be developed to improve overall survival (OS) of these patients, and cancer vaccines have been discussed as a promising approach against refractory sarcomas because of the expressions of tumor-associated antigens (TAA) on sarcoma tissues.[1,2,5–9] Nevertheless, there have been few clinical trials of cancer vaccination for refractory sarcoma patients. One of the hurdles could be the fact that there are many sarcoma subtypes along with different human leukocyte antigen (HLA)-types.

We have developed a novel regime of personalized peptide vaccination (PPV), in which vaccine antigens are selected and administered from a pool of 31 different peptide candidates based on the pre-existing IgG responses specific to peptides before vaccination.[10–13] In previous studies, the PPV was feasible for the vast majority of cancer patients with different HLA-types.[10–13] A recently conducted randomized clinical trial of PPV in advanced prostate cancer patients showed a favorable clinical outcome in the vaccinated group.[14] In the present study, we addressed whether PPV treatment would be feasible for refractory bone and soft tissue sarcoma patients with various HLA-types by conducting a small-scale phase II study.

Materials and Methods

TAA and HLA-class I expressions in sarcoma tissues. The expressions of 15 different TAA, from which the vaccine peptides used for PPV were derived, were examined by immunohistochemistry (IHC) in 26 sarcoma tissues (11 leiomyoarcoma, five synovial sarcoma, five malignant fibrous histiocytoma, and five liposarcoma) as previously reported.[15] The expression of HLA-class I was also examined by IHC in the 26 sarcoma tissues by using an anti-HLA-class I antibody (murine monoclonal, clone EMR8-5; Abcam, Cambridge, UK).

Patients. Patients with histological diagnosis of bone and soft tissue sarcoma were eligible for inclusion in the present study. All patients were required to have evaluable recurrent and/or metastatic tumors at the time of entry. Patients whose general condition was tolerable for chemotherapy or radiotherapy were eligible only after the failure of these therapies. Patients, who had poor general conditions intolerable for chemotherapy or radiotherapy, or refused them, were also eligible. All patients were required to show positive IgG responses to at least two of the 31 different vaccine candidate peptides, as reported previously.[10–12] Other inclusion criteria were as follows: age between 20 and 80 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; positive status for the HLA-A2, -A24, -A3 supertypes (A3, A11, A31, or A33), or -A26 types; life expectancy of at least 12 weeks; and adequate hematologic, hepatic, and renal function. Exclusion criteria included pulmonary, cardiac, or other systemic diseases; an acute infection; a history of severe allergic reactions; pregnancy or nursing; and other inappropriate conditions for enrollment as judged by clinicians. Patients with a lymphocyte count of <1000/μL were excluded from the study, since we previously reported that pre-vaccination lymphocytopenia (<1000 cells/μL) is an unfavorable factor for OS in cancer patients receiving PPV.[16,17] The protocol was approved by the Kurume University Ethical Committee and registered in the UMIN Clinical Trials Registry

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(UMIN#000002282). All patients were given a full explanation of the protocol and provided their informed consent before enrollment.

Clinical protocol. This was a phase II study to evaluate the safety and immunological responses in refractory bone and soft tissue sarcoma patients under PPV. Thirty-one peptides, whose safety and immunological effects for other types of cancer were confirmed in previously conducted clinical studies,[11–14] were used for vaccination (12 peptides for HLA-A2, 16 peptides for HLA-A24, nine peptides for HLA-A3 supertypes [-A3, -A11, -A31, and -A33], and four peptides for HLA-A26) (Table S1). These peptides were prepared under the condition of Good Manufacturing Practice (GMP) by the PolyPeptide Laboratories (San Diego, CA, USA) and American Peptide Company (Vista, CA, USA). Peptides for vaccination to individual patients were selected in consideration of the pre-existing host immunity before vaccination, as assessed by the titers of IgG specific to each of the 31 different vaccine candidates.

A maximum of four peptides (3 mg/each peptide), which were selected based on the results of HLA typing and peptide-specific IgG titers, were subcutaneously administrated with incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France) once a week for six consecutive weeks. After the first cycle of six vaccinations, up to four antigen peptides, which were re-selected according to the titers of peptide-specific IgG at the 6th vaccination, were administered every 2 weeks. During the PPV, patients were allowed to receive combination therapies, such as chemotherapies or radiotherapies. Since the frequency of bone and soft tissue sarcomas had been low, we enrolled the patients treated both with and without combination therapies to facilitate the enrolment. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI-CTC Ver.-3.0). Complete blood counts and serum biochemistry tests were performed every six vaccinations. The clinical responses were determined by the Response Evaluation Criteria in Solid Tumors (RECIST) in the vaccinated patients. The RECIST-based clinical responses were evaluated every six vaccinations by radiological findings of computed tomography (CT) scan and/or magnetic resonance imaging (MRI), and the

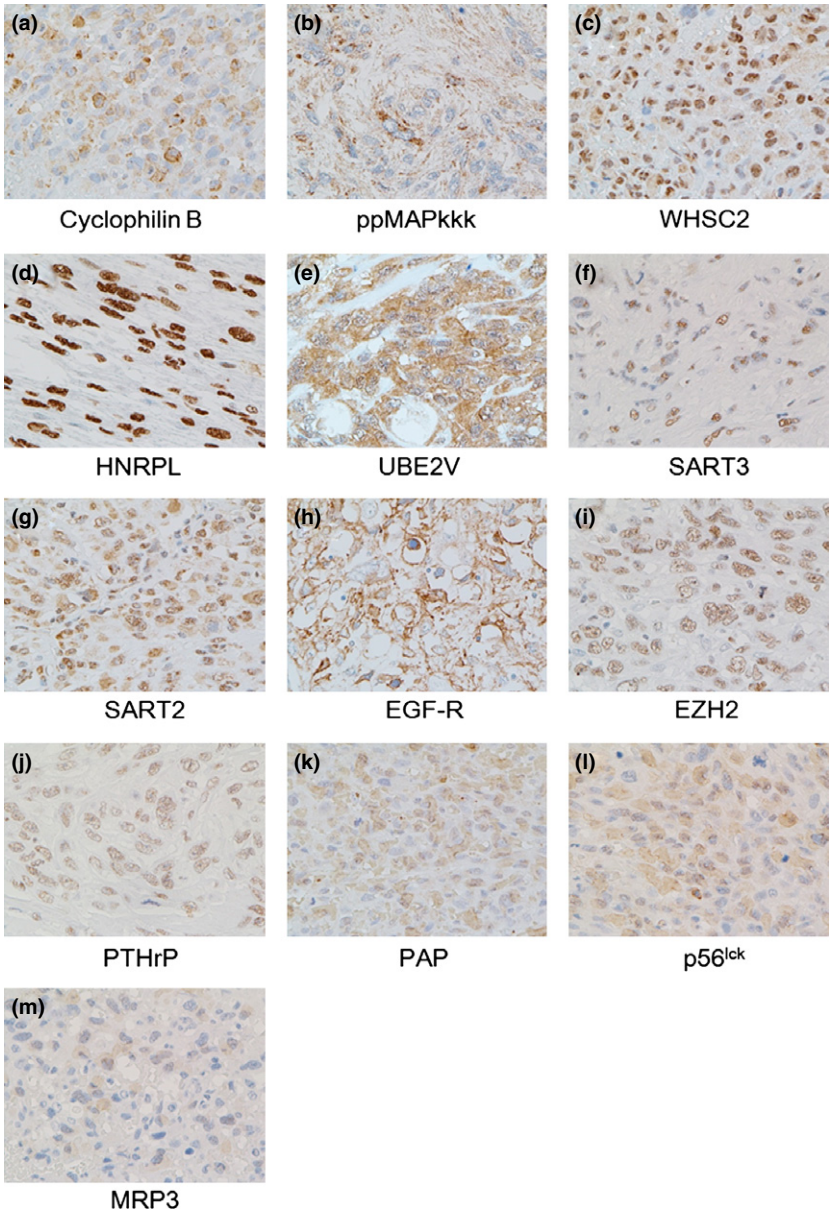

Fig. 1. Expressions of tumor-associated antigens (TAA) in soft tissue sarcoma tissues. Expressions of TAA were examined by immunohistochemistry in soft tissue sarcoma tissues. Thirteen out of the 15 TAA were expressed at different frequencies in soft tissue sarcoma tissues. Representative results are shown (a–m: ×400). The remaining two prostate-related antigens, PSA and PSMA, were not detectable by immunohistochemistry in sarcoma tissues tested (not shown).

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best overall responses during PPV treatment were shown. For the patients who did not complete the first cycle of six vaccinations, the newest radiological findings were evaluated.

Measurement of humoral and cellular immune responses and inflammatory cytokine and markers. Humoral immune responses specific to each of the 31 peptide candidates were determined by peptide-specific IgG levels using the Luminex system (Luminex, Austin, TX, USA), as previously reported.[17] If the titers of peptide-specific IgG to at least one of the vaccinated peptides in the post-vaccination plasma were more than two-fold higher than those in the pre-vaccination plasma, the changes were considered to be significant as reported previously.[11–16] Cellular immune responses specific to the vaccinated peptides were evaluated by interferon (INF)-γ ELISPOT using PBMCs as reported previously.[11–16] As a control, cellular immune responses specific to CEF peptides (MABTECH, Cincinnati, OH, USA), a mixture of virus-derived CTL epitopes, were also examined. Inflammatory cytokine and markers, including, interleukin-6 (IL-6), C-reactive protein (CRP), and serum amyloid A (SAA), in plasma samples were also examined by ELISA as reported previously.[15]

Statistical analysis. The two-sided Wilcoxon test was used to examine differences between pre- and post-vaccination measurements. *P*-values <0.05 were considered to be statistically significant. Progression-free survival (PFS) or OS were calculated from the date of the first vaccination until the date of disease progression or death, respectively, or the last date when the patient was known to be alive. Predictive factors for OS

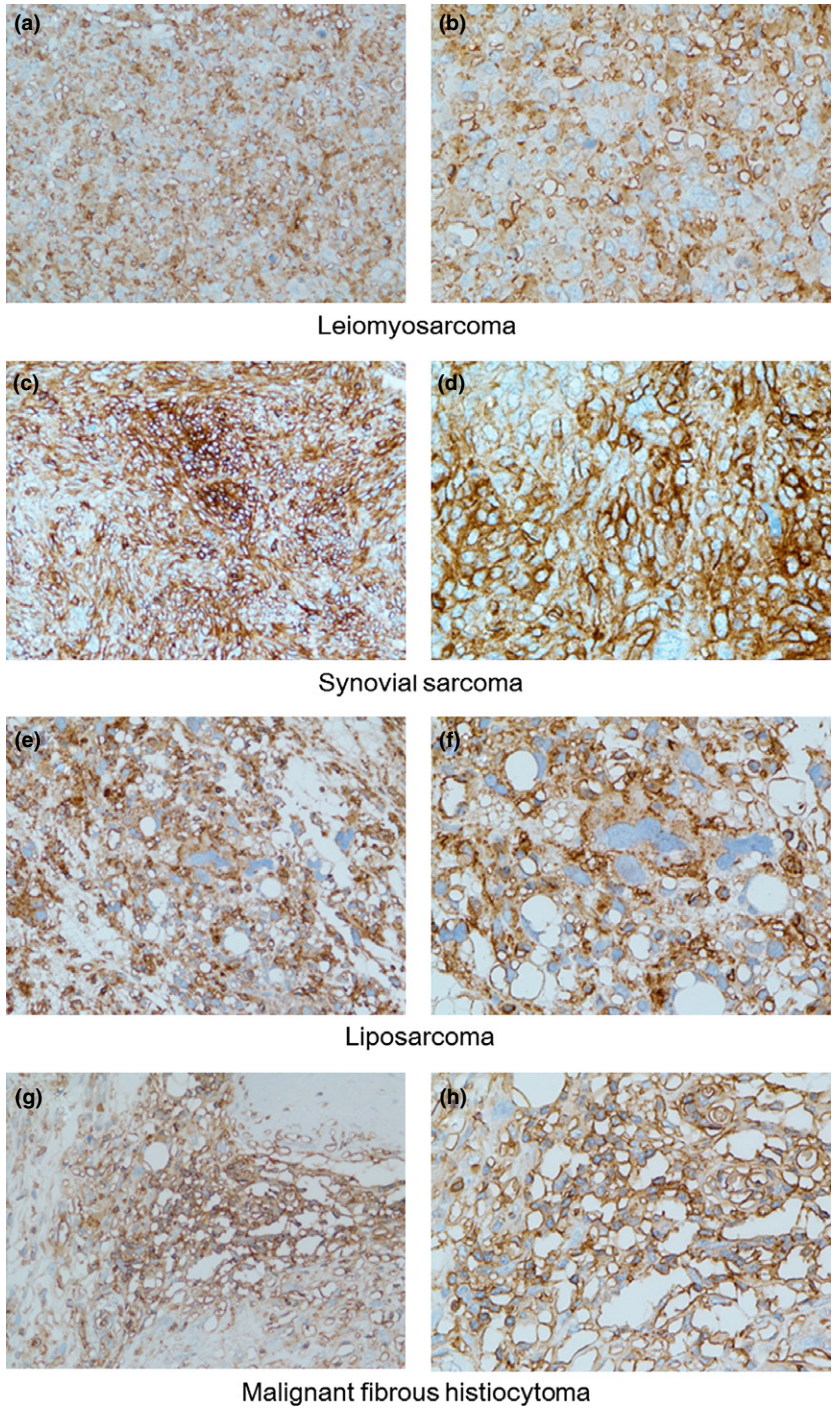

Fig. 2. Expressions of HLA-class I in soft tissue sarcoma tissues. Expressions of HLA-class I were examined by immunohistochemistry in different subtypes of soft tissue sarcoma tissues. (a,b) leiomyosarcoma (a, ×200; b, ×400). (c,d) synovial sarcoma (c, ×200; d, ×400). (e,f) liposarcoma (e, ×200; f, ×400). (g,h) malignant fibrous histocytoma (g, ×200; h, ×400).

Table 1. Characteristics of the enrolled patients with refractory sarcoma

No.	HLA type	Sex	Age	Pathology	Stage	PS	Previous radiotherapy	No. of previous chemotherapy	Periods of chemotherapy	Disease location	No. of vaccination	Combined therapy	Treatment response	PFS	Survival times
1	A2/A24	M	64	Osteosarcoma	rec.	0	+	2	23.0	Lung	12	–	PD	4.8	9.7
2	A2/A11	M	31	Malignant neurinoma	rec.	0	+	1	6.8	Lung, Mediastinal LN	17	–	SD	6.8	7.6
3	A2/A24	M	60	MFH	rec.	0	–	–	–	Lung, Inguinal LN	6	–	PD	2.0	2.3†
4	A2/A24	F	30	Synovial sarcoma	rec.	0	+	4	32.0	Lung	6	Radiotherapy	PD	1.8	4.6
5	A2/A26	M	54	Synovial sarcoma	IV	0	–	1	10.0	Mediastinal LN	6	–	SD	33.0	35.0†
6	A24/A33	F	74	Liposarcoma	rec.	1	+	–	–	Humerus, Thoracic vertebra	14	–	SD	7.4	9.2†
7	A11/A33	F	49	Leiomyosarcoma	IV	0	–	2	3.8	Lung, Liver, Sacrum	15	–	PD	4.5	10.0
8	A33	F	23	Osteosarcoma	rec.	0	–	4	6.9	Lung, Hilar LN	4	–	PD	1.6	2.2
9	A24/A33	M	43	Epithelioid sarcoma	rec.	1	+	–	–	Parasternal LN, Pleura	12	–	SD	7.0	25.0†
10	A24	F	33	Leiomyosarcoma	rec.	0	–	3	17.0	Lung, Liver, Peritoneum	12	GEM + DTX	PD	4.4	9.6
11	A24/A26	F	62	Liposarcoma	rec.	0	–	2	2.7	Liver, Retroperitoneum	4	–	PD	1.5	7.2
12	A24/A26	F	55	Clear cell sarcoma	rec.	1	–	3	4.6	Lung, Intraabdominal LN	6	–	PD	3.5	21.0†
13	A24	M	73	MFH	rec.	0	–	–	–	Lung, Liver	11	–	SD	11.0	11.0
14	A26/A30	F	38	MFH	rec.	1	–	2	2.3	Liver, Retroperitoneum	3	–	PD	1.4	2.5
15	A24	F	75	Leiomyosarcoma	rec.	0	–	–	–	Liver, Lumbar vertebra, Sacrum, Femur	12	–	PD	4.4	6.2
16	A2/A24	M	64	Chondrosarcoma	rec.	0	–	–	–	Lung, Pleura	14	–	SD	5.7	5.7†
17	A2	F	63	Leiomyosarcoma	rec.	0	+	3	24.0	Lung, Subcutaneous tissue	6	GEM	PD	1.8	4.7
18	A2/A11	M	71	Osteosarcoma	rec.	0	–	–	–	Local recurrence, Lung	11	–	PD	3.5	4.1†
19	A2/A26	M	45	Alveolar soft part sarcoma	rec.	0	+	3	65.0	Lung, Brain	10	Radiotherapy CPA	PD	3.5	3.6†
20	A11/A31	M	27	Synovial sarcoma	rec.	0	–	2	8.9	Lung, Liver, Retroperitoneum	6	Sorafenib	PD	1.6	3.2

†Survivor. CPA, cyclophosphamide; DTX, docetaxel; F, female; GEM, gemcitabine; LN, lymph nodes; M, male; MFH, malignant fibrous histocytoma; PS, performance status; PD, progressive disease; PFS, progression-free survival; rec., recurrent status; SD, stable disease.

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Table 2. Toxicities in vaccinated patients with refractory sarcoma

	Grade 1	Grade 2	Grade 3	Grade 4
Injected site reaction	13	7		
Constitutional symptom				
Fever	3			
Malaise	1			
Gastrointestinal				
Nausea	1			
Respiratory				
Dyspnea	1			
Blood/Bone marrow				
Anemia	11	1	2	
Leucocytopenia	7			
Neutropenia				
Lympocytopenia	11	2	1	
Thorombocytopenia			1	
Laboratory				
AST elevation	1	1		
ALT elevation	3			
Creatinine elevation	2			
Hypoalbuminea	9		1	

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

were evaluated by univariate analysis with the Cox proportional hazards regression model.

Results

TAA and HLA-class I expressions in sarcoma tissues. Figures 1 and 2 show the representative data of TAA and HLA-class I expressions in soft tissue sarcoma tissues determined by IHC. Thirteen out of 15 TAA were expressed at different frequencies in soft tissue sarcoma tissues, as follows: Cyclophilin B, 20/26 (77%); ppMAPkkk, 15/26 (58%); WHSC2, 23/26 (88%); HNRPL, 25/26 (96%); UBE2V, 17/26 (65%); SART3, 26/26 (100%); SART2, 26/26 (100%); EGF-R, 17/26 (65%); EZH2, 13/26 (50%); PTHrP, 9/26 (35%); PAP, 13/26 (50%); $p56^{lck}$, 7/26 (27%); MRP3, 3/26 (12%). However, the remaining two prostate-related antigens (PSA and PSMA) were not detectable by IHC (data not shown). HLA-class I was expressed in 25 of 26 various subtypes of sarcoma tissues examined, except for one synovial sarcoma tissue.

Patients' characteristics. Between August 2009 and May 2012, 20 patients with refractory bone and soft tissue sarcoma (leiomyosarcoma, $n = 4$; osteosarcoma, $n = 3$; synovial sarcoma, $n = 3$; malignant fibrous histiocytoma, $n = 3$; liposarcoma, $n = 2$; chondrosarcoma, $n = 1$; malignant neurinoma, $n = 1$; epithelioid sarcoma, $n = 1$; clear cell sarcoma, $n = 1$; alveolar soft part sarcoma, $n = 1$), were enrolled in this study. Table 1 shows the clinicopathological characteristics of the 20 patients (10 male and 10 female). Performance status at the time of enrollment was grade 0 ($n = 16$) or grade 1 ($n = 4$). Five patients (#6, #15, #18, #20, and #22; all ≥60-year-old) had received neither chemotherapy nor radiotherapy because they refused these treatments or their general condition was not tolerable for them. The median age was 55 years, ranging from 23 to 75 years. Thirteen patients had received unsuccessful chemotherapy. The median duration of previous chemotherapy was 8.9 months, ranging from 2.3 to 65 months. Patients received one ($n = 2$), two ($n = 5$), three ($n = 4$), or four ($n = 2$) chemotherapy regimens, and the median number of chemotherapy regimens was two. The median duration from the first recurrence to the PPV was 13 months, ranging from 1 to 76 months. Seven patients had received unsuccessful radiotherapy. Of the total 20 patients, 17 completed the first cycle of vaccinations, whereas the remaining three patients failed

Table 3. Immune responses to the vaccine peptides

Patient No.	Peptide	IgG response†		CTL response‡	
		Before	1st	Before	1st
1	CypB-129	123	137	0	141
	Lck-246	15	15	0	0
	PAP-213	76	5263	0	590
	Lck-486	71	6772	0	0
	CEF			0	0
2	UBE2V-43	18	7691	0	271
	HNRPL-140	40	54	0	0
	Lck-449	70	0	0	0
	WHSC2-103	28	0	0	0
	CEF			1240	928
3	UBE2V-85	17	22	72	0
	SART3-302	110	27 557	0	181
	MRP3-503	33	76	0	156
	PSMA-624	32	25	0	0
	CEF			0	0
4	SART2-93	40	0	0	0
	MRP3-503	52	87	0	101
	MRP3-1293	1111	1714	296	0
	SART2-161	33	36	0	0
	CEF			320	499
5	WHSC2-103	1109	4965	0	581
	WHSC2-141	806	83 798	0	0
	SART3-302	812	2052	0	473
	SART3-309	634	1249	0	256
	CEF			998	0
6	PAP-213	62	49	0	328
	MRP3-503	12	0	0	0
	SART2-161	34	27	0	0
	Lck-488	133	102	0	0
	CEF			0	0
7	Lck-449	20	18	0	0
	CypB-129	31	33	0	0
	CEF			0	0
8	Lck-449	46	NA	0	NA
	CypB-129	43	NA	0	NA
	WHSC2-103	14	NA	0	NA
	CEF			0	NA
9	Lck-208	36	0	0	0
	EGF-R-800	152	88	0	0
	Lck-486	27	14 962	0	0
	EZH2-735	64	2359	0	0
	CEF			0	0
10	PAP-213	68	54	0	79
	PSA-248	16	1059	0	0
	CEF			0	0
11	SART2-93	12	NA	0	NA
	PAP-213	81	NA	0	NA
	PSA-248	12	NA	0	NA
	Lck-486	28	NA	0	NA
	CEF			0	NA
12	SART2-93	16	23	0	0
	PSA-248	99	2501	0	0
	Lck-486	28	11 642	0	0
	Lck-488	48	3586	0	0
	CEF			0	0
13	SART2-93	45	38	0	194
	SART3-109	44	23	0	0
	Lck-486	56	45	0	365
	Lck-488	59	51	0	0
	CEF			432	134

Table 3. (continued)

Patient No.	Peptide	IgG response†		CTL response‡	
		Before	1st	Before	1st
14	SART3-109	20	NA	0	NA
	WHSC2-103	15	NA	0	NA
	CEF			0	NA
15	SART2-93	3348	2612	0	0
	PSA-248	189	12 486	0	0
	Lck-488	94	3314	0	103
	PTHrP-102	47	69	0	0
	CEF			0	0
16	WHSC2-103	1000	1115	409	2773
	SART3-109	1665	1774	0	0
	MRP3-1293	298	265	0	0
	Lck-488	225	226	0	2084
	CEF			4558	3699
17	CypB-129	93	55	0	0
	WHSC2-103	110	52	0	0
	HNRPL-501	158	5472	0	0
	WHSC2-141	116	78	0	0
	CEF			5256	8402
18	Lck-422	11	0	0	713
	SART3-309	13	16	0	0
	SART3-734	783	6089	0	1418
	Lck-90	21	25	0	0
	CEF			1631	2640
19	ppMAPkkk-432	132	148	0	0
	HNRPL-140	60	0	0	417
	SART3-302	68	400	0	347
	SART3-109	259	364	0	0
	CEF			3459	2459
20	SART3-734	369	328	0	0
	Lck-90	64	53	0	0
	CypB-129	48	40	0	0
	WHSC2-103	43	75	0	0
	CEF			1486	2483

†Values indicate the fluorescence intensity unit (FIU) of plasma IgG reactive with the corresponding peptides before and after the 1st cycle of vaccination. The augmented IgG responses are underlined.
‡Values indicate the number of spots per 10^5 peripheral blood mono-nuclear cells (PBMCs) reactive with the corresponding peptides in IFN-γ ELISPOT assay before and after the 1st cycle of vaccinations. When the number of spots was <30 per 10^5 PBMCs, the data are shown as "0". The augmented T cell responses are underlined. CEF, a mixture of virus-derived CTL epitopes; NA, not assessed.

due to rapid disease progression. The median number of vaccinations was 10, ranging from 3 to 17. During the PPV, three patients were treated in combination with chemotherapies, and two patients were treated with radiotherapies, while the remaining 15 patients had no combination therapies.

Toxicities. Grade 1 or 2 dermatological reaction at the injection sites was observed in all cases (Table 2). Anemia (n = 14), lymphocytopenia (n = 14), and hypoalbuminemia (n = 10) were observed frequently. Grade 3 adverse events included anemia (n = 2), lymphocytopenia (n = 1), thrombocytopenia (n = 1), and hypoalbuminemia (n = 1). According to evaluation by the independent safety evaluation committee in this trial, all of these Grade 3 adverse events were concluded to be not directly associated with the PPV, but with the disease progression.

Immune responses to the vaccinated peptides. Both humoral and cellular immune responses specific to the vaccinated peptides were analyzed in blood samples before and after vaccination (Table 3). Plasma samples were collected from 20 and 17 patients before and at the 6th vaccinations, respectively.

Table 4. Changes of inflammatory cytokine and markers

Patient No.	IL-6 (pg/mL)		CRP (mg/dL)		SAA (mg/dL)	
	Before	1st	Before	1st	Before	1st
1	4	3	7.2	8.5	23.0	54.0
2	4	3	0.7	0.7	0.0	2.2
3	0	4	10.0	11.0	180.0	177.0
4	0	7	2.3	13.0	5.6	183.0
5	0	0	0.7	2.2	2.0	1.7
6	6	8	6.6	10.0	67.0	188.0
7	2	3	0.7	1.5	8.4	13.0
8	42	NA	16.0	NA	118.0	NA
9	0	6	4.3	1.0	80.0	139.0
10	0	6	0.0	10.0	0.5	94.0
11	0	NA	13.0	NA	110.0	NA
12	0	0	8.6	0.7	126.0	26.0
13	3	5	4.2	6.6	83.0	129.0
14	60	NA	8.7	NA	159.0	NA
15	5	10	7.0	6.7	148.0	41.0
16	0	0	0.5	1.5	0.0	3.0
17	3	5	6.3	8.7	32.0	148.0
18	9	7	4.0	2.7	12.0	4.2
19	2	0	8.7	3.6	144.0	136.0
20	11	8	2.4	8.0	160.0	58.0

CRP, C-reactive protein; IL-6, interleukin-6; NA, not assessed; SAA, serum amyloid A.

Plasma samples from three patients, who failed to complete the first cycle of six vaccinations due to disease progression, were unavailable. For the monitoring of humoral immune responses, peptide-specific IgG reactive to each of the 31 different peptides, including both vaccinated and non-vaccinated peptides, were measured by bead-based multiplex assay. The numbers of peptides used for the first cycle of vaccinations were 2, 3, or 4 in 3, 1 or 16 patients, respectively (Table 3). Augmentation of the IgG responses specific to at least one of the vaccinated peptides after vaccination was observed in 11 of 17 patients (64.7%). We also evaluated epitope spreading by comparing the peptide-specific IgGs to non-vaccinated peptides in plasma before and after vaccination. As a result, 12 of 17 patients (70.6%) showed epitope spreading to at least one of the non-vaccinated peptides (Table S2).

Cellular immune responses to the vaccinated peptides were assessed by INF-γ ELISPOT assay (Table 3). Antigen-specific CTL responses were detectable in only three of 20 patients before vaccination. In contrast, augmentation of the CTL responses specific to at least one of the vaccinated peptides after vaccination was observed in 12 of 17 patients (70.6%). We also tested CTL responses to CEF peptides, a mixture of virus-derived CTL epitopes, as a control. Cytotoxic T-lymphocyte responses to CEF peptides were observed in 9 of 20 (45%) patients before vaccination and 8 of 17 (47%) patients after vaccination, respectively.

Collectively, eight patients showed both increased CTL and IgG responses to the vaccinated peptides, 16 of 17 patients showed either increased CTL or IgG responses, and the remaining one patient showed neither CTL nor IgG boosting. There were no significant differences in increase in CTL or IgG responses between the patients treated with PPV alone (n = 12) and those treated with combination therapies (n = 5) (P = 0.794 and P = 0.543, respectively; χ^2 test).

Inflammatory cytokine and markers. We measured inflammation cytokine and markers, including IL-6, CRP and SAA, in the plasma before and at the 6th vaccination. IL-6 was detectable in 12 patients before vaccination with a median of 2.5 pg/mL, ranging from 0 to 60 pg/mL. IL-6 levels were increased,

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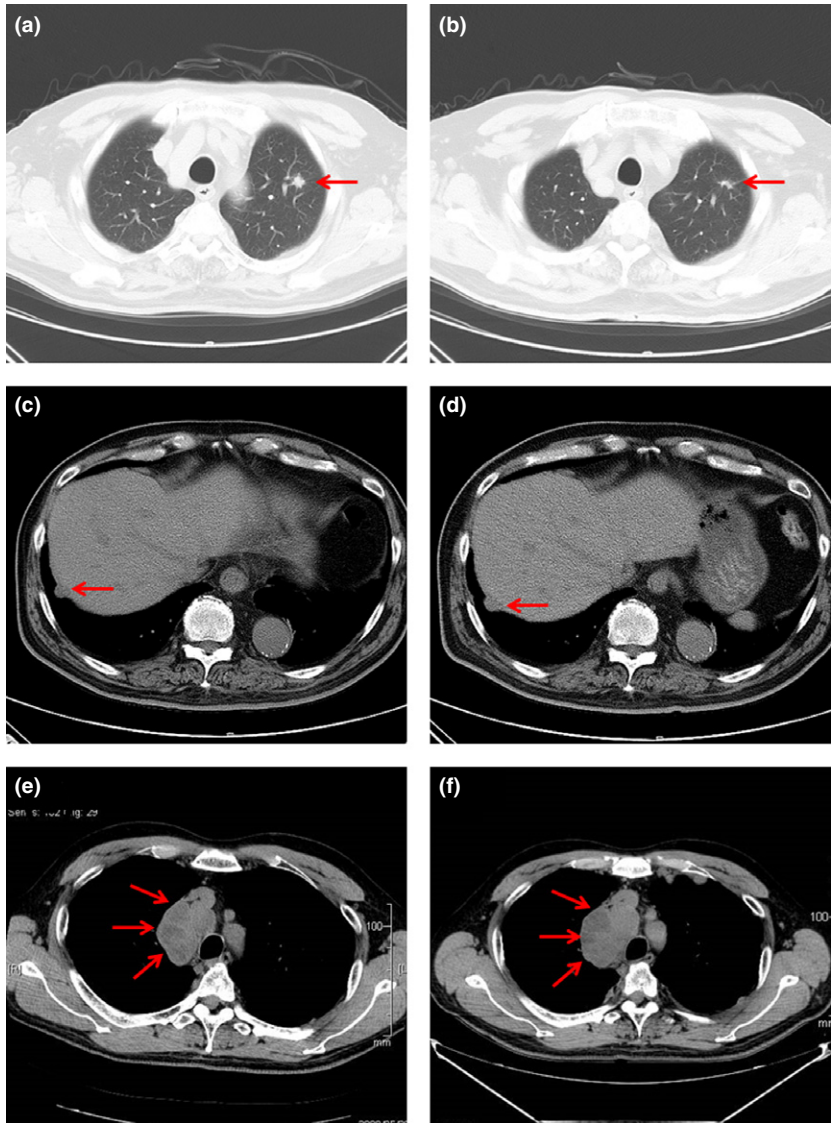

Fig. 3. Clinical responses to personalized peptide vaccination (PPV). (a–d) Computed tomography findings of one of stable disease (SD) cases before and after the 6th vaccination. At 4 months after the first vaccination, the lung metastasis was remarkably reduced in size, but the liver metastasis showed no changes in size. (e,f) Computed tomography findings of another SD case before and after the 6th vaccination. A huge mediastinal tumor showed no increase in size for a period of 34 months after the first vaccination.

decreased, or unchanged in nine, five, or three patients tested, respectively (Table 4). A significant increase was observed in IL-6 levels after vaccination ($P = 0.034$, Wilcoxon test).

An inflammation marker, CRP, was detectable in the pre-vaccination plasma of 19 patients with a median value of 5.3 mg/dL (ranging from 0 to 16 mg/dL). Plasma CRP levels were increased, decreased, or unchanged in 11, 5, or 1 patients, respectively (Table 4). Another inflammation marker, SAA, was also detected in the pre-vaccination plasma of 18 patients with a median value of 73.5 mg/dL (ranging from 0 to 180 mg/dL). Plasma SAA levels were increased or decreased in 10 or 7 patients, respectively (Table 4). There was a significant increase in the levels of CRP after vaccination ($P = 0.027$, Wilcoxon test), while there was no significant difference in the levels of SAA between before and after vaccination ($P = 0.178$, Wilcoxon test).

Clinical responses and biomarker analysis. Best clinical responses were evaluated by radiological findings. There were no complete response (CR), no partial response (PR), six stable disease (SD), and 14 progressive disease (PD; Table 1). Computed tomography findings of two SD cases before and after the 6th vaccination are shown in Figure 3. One of the SD cases (case #13 in Table 1) was a 72-year-old man with recurrent malignant fibrous histiocytoma treated with PPV alone. At 4 months after the first vaccination, the lung metastasis was remarkably reduced in size (Fig. 3a,b), but the liver metastasis showed no changes in size (Fig. 3c,d). Another SD case (case #5 in Table 1) was a 54-year-old man with advanced synovial sarcoma, who was also treated with PPV alone. He had a huge mediastinal tumor, which showed no increase in size for a period of 34 months after the first vaccination (Fig. 3e,f). The cellular immune responses to vaccinated peptides were well boosted in both cases, while IgG responses to vaccine peptides were not boosted in one of them (case #13 in Table 3).

The median survival time (MST) and median progression-free survival time (MPFST) of the 20 patients was 9.6 months (95% confidence interval [CI], 4.7–11.0 months) and 4 months (95% CI, 1.8–6.8 months; Fig. 4). The MST and MPFST of the five patients treated with PPV plus combination therapies were significantly worse than those of the 15 patients treated with PPV alone (MST, 4.7 *vs* 10 months, $P = 0.037$; MPFS, 1.8 *vs* 4.5 months, $P = 0.045$; Fig. S1a,b). Under these circumstances, the Cox proportional hazards model was used to identify prognostic factors for OS. In the univariate analysis with pre-vaccination data, lymphocytopenia and higher levels of IL-6 were unfavorable factors for OS ($P = 0.020$ and $P = 0.014$, respectively). To better understand their involvement, a log-rank test was used for the statistical analysis. The

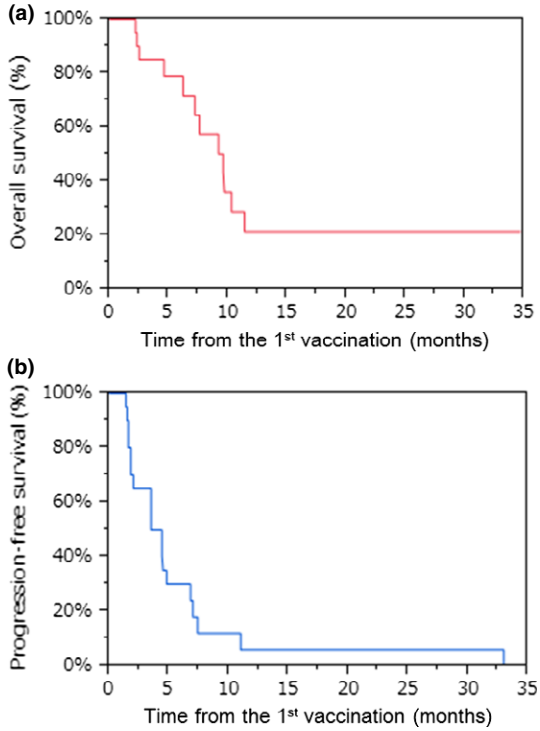

Fig. 4. Overall survival and progression-free survival. (a) Median survival time of the 20 patients with refractory sarcomas under personalized peptide vaccination (PPV) was 9.6 months (95% confidence interval [CI], 4.7–11.0 months). (b) Median progression-free survival time of the 20 patients with refractory sarcomas under PPV was 4 months (95% CI, 1.8–6.8 months).

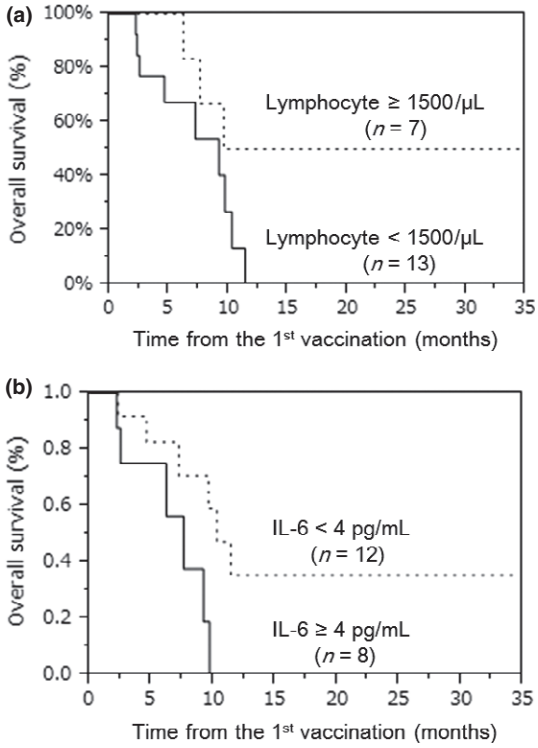

Fig. 5. Pre-vaccination biomarker analysis. (a) The patients with lymphocytopenia (<1500/μL) in the pre-vaccination samples showed shorter overall survival (OS) (P = 0.071, Log-rank test). (b) The patients with higher levels of interleukin-6 (IL-6) (≥4 pg/mL) in the pre-vaccination samples showed shorter OS (P = 0.035, Log-rank test).

patients with lymphocytopenia (<1500/μL; P = 0.071) or higher levels of IL-6 (≥4 pg/mL; P = 0.035) in the pre-vaccination samples showed shorter OS (Fig. 5). The univariate analysis with post-vaccination data at the time of the 6th vaccination showed that the epitope spreading to at least three of the non-vaccinated peptides was the favorable factor for OS (P = 0.020). A log-rank test also showed that the presence of epitope spreading to at least three of the non-vaccinated peptides in the post-vaccination samples showed longer OS (P = 0.020; Fig. 6).

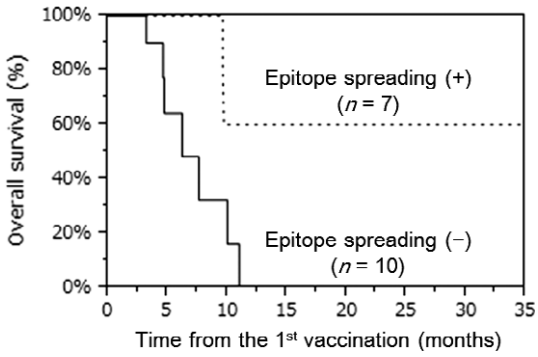

Fig. 6. Post-vaccination biomarker analysis. The patients with epitope spreading in the post-vaccination samples showed longer overall survival (OS) (P = 0.020, Log-rank test).

Discussion

By IHC analysis, 13 out of 15 TAA, from which the vaccine peptides used for PPV were derived, were expressed in all subtypes of sarcoma tissues examined (leiomyoarcoma, synovial sarcoma, malignant fibrous histiocytoma, and liposarcoma). In addition, HLA-class I was expressed in almost all of the sarcoma tissues examined, except for one synovial sarcoma tissue. These results suggest that these TAA could be used as a target of immunotherapy for refractory sarcoma patients. In contrast, two prostate-related antigens, PSA and PSMA, whose expressions were primarily restricted to prostate cancers, were not detectable by IHC analysis in sarcoma tissues. Nevertheless, only five among 74 peptides that were vaccinated to 20 patients at the first cycle were derived from either PSA (four cases) or PSMA (one case; Table 3). Considering there was no expression of prostate-related antigens in sarcoma tissues examined, PSA- and PSMA-derived peptides should be selected only for patients who have no IgG responses to the other peptides in the next PPV trial for sarcoma patients, as reported previously.[11]

The phenotypes of HLA-class IA antigens of the 20 patients were very diverse, with the HLA-A24, -A2, -A26, -A11, -A33, -A30, and -A31 types occurring in 11, 9, 5, 4, 3, 1, and 1 case, respectively. These frequencies are expected based on previous reports in the Japanese population.[18] Importantly, peptide-specific CTL or IgG boosting after vaccination was observed in the majority of patients tested, regardless of the different histological types of sarcoma cells and different HLA-types. It is also of note that only 3 of 20 patients showed peptide-specific CTL responses in pre-vaccination PBMCs, but CTL responses became detectable in 12 of 17 patients after vaccination. On the contrary, the frequencies of CTL responses to virus-related peptides were not different between the pre- (9 of 20 cases) and post-vaccination (8 of 17 cases) samples. These results suggest that immune boosting was really restricted to the vaccinated peptides, and did not inhibit cellular immunity to infectious viruses.

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In addition, no severe adverse events related to PPV were observed. These findings suggest that PPV using 31 vaccine peptide candidates could be feasible for the vast majority of sarcoma patients at least in Japan, and probably also worldwide, since the seven different HLA-types mentioned above along with the HLA-A3 type would be expected to cover the vast majority of sarcoma patients.

In the pre-vaccination samples, the lymphocytopenia and higher levels of IL-6 were inversely correlated with OS. IL-6 is a multifunctional cytokine that regulates various aspects of immune responses, acute phase reactions, and hematopoiesis.[19,20] In addition, IL-6 has recently been reported to be one of the critical cytokines for inducing suppressive immune cell subsets, such as myeloid-derived suppressor cells and Th17, which are known to negatively affect anti-tumor immunity.[21–23] It thus might be possible that high levels of IL-6 innibit immune responses to cancer vaccines. In the post-vaccination samples, the presence of epitope spreading was well correlated with OS, whereas there were no significant correlations between epitope spreading to some particular antigens, such as HNRPL-501, SART2-93, MRP3-1293, and PSMA-624 (Table S2), and good clinical outcomes. Neither CTL nor IgG boosting correlated with OS in this study, although we reported that both CTL and IgG boosting were well correlated with longer OS in our previous clinical trial for other types of cancers.[16] This discrepancy could be related to the fact that immunological boosting was observed in the majority of sarcoma patients (>70%) or to the fact that only 20 patients were tested in this study.

In the present study, PPV has shown promising clinical benefits in refractory sarcoma patients with a MST of 9.6 months and a MPFST of 4 months. Previously, second-line palliative chemotherapy for advanced soft tissue sarcoma patients was reported to show a MST of 8 months and a 23% PFST at 6 months.[24] In addition, best supportive care for elderly advanced soft tissue sarcoma patients was shown to reveal a MST of 5.3 months.[25] Compared to these previous studies in patients with similar disease conditions, our results suggest that PPV could be an attractive therapeutic modality for refractory sarcoma patients because of the safety and potential survival benefits. Of note, combined treatments with chemotherapy or radiotherapy did not affect antigen-specific immune responses, but deteriorated PFST and OS in patients receiving PPV, indicating that combined treatments would not be beneficial, although the numbers of patients were too small to conclude in this study.

In conclusion, PPV could be feasible for the vast majority of refractory sarcoma patients because of the safety and higher rates of immunological responses regardless of the presence of different sarcoma subtypes and various HLA-types.

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Disclosure Statement

Kyogo Itoh is a Chief Scientific Advisor for the Green Peptide Company, Ltd. The other authors declare that they have no competing interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Survival curves for patients treated with personalized peptide vaccination (PPV) with or without combination therapies.

Table S1. Information of peptide candidates used for personalized peptide vaccination (PPV).

Table S2. Epitope spreading status in sarcoma patients after personalized peptide vaccination (PPV).

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