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

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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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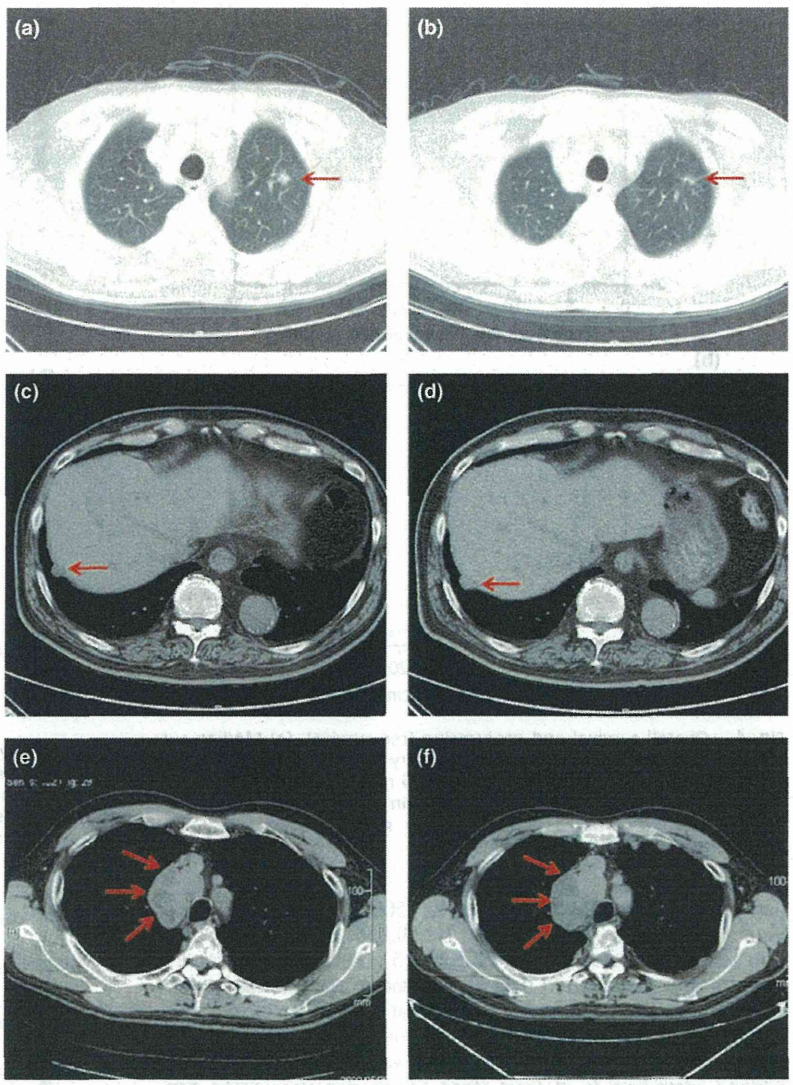

Fig. 3. Clinical responses to personalized peptide vaccination (PPV). (a–d) Computed tomography findings of one of stable disease (SD) cases before and after the 6th vaccination. At 4 months after the first vaccination, the lung metastasis was remarkably reduced in size, but the liver metastasis showed 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

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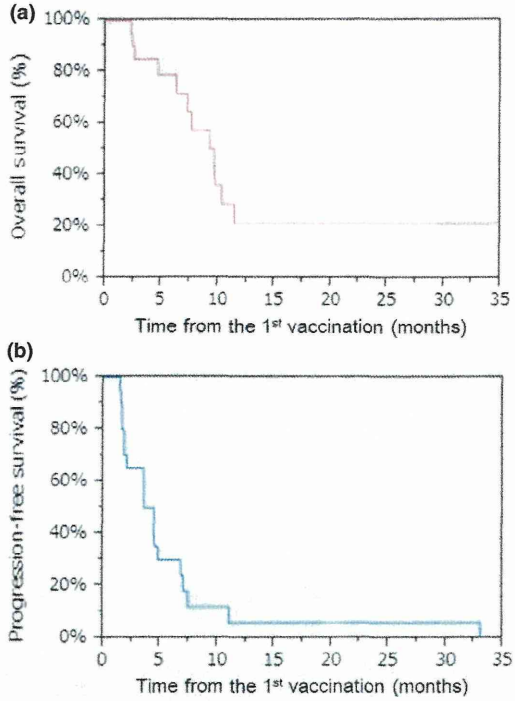

Fig. 4. Overall survival and progression-free survival. (a) Median survival time of the 20 patients with refractory sarcomas under personalized peptide vaccination (PPV) was 9.6 months (95% confidence interval [CI], 4.7–11.0 months). (b) Median progression-free survival time of the 20 patients with refractory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

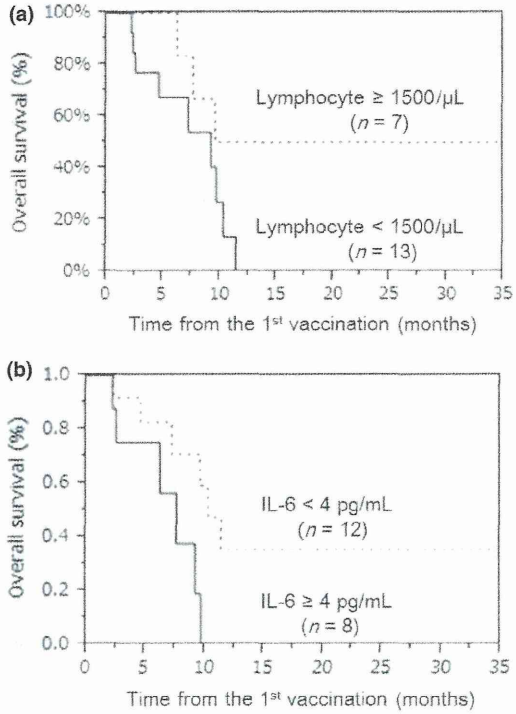

Fig. 5. Pre-vaccination biomarker analysis. (a) The patients with lymphocytopenia (<1500/μL) in the pre-vaccination samples showed shorter overall survival (OS) (P = 0.071, Log-rank test). (b) The patients with higher levels of interleukin-6 (IL-6) (≥4 pg/mL) in the pre-vaccination samples showed shorter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

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.

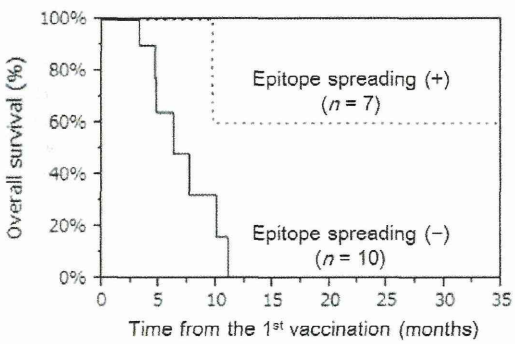

Fig. 6. Post-vaccination biomarker analysis. The patients with epitope spreading in the post-vaccination samples showed longer overall survival (OS) (P = 0.020, Log-rank test).

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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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Research Article

Juzentaihoto Failed to Augment Antigen-Specific Immunity but Prevented Deterioration of Patients' Conditions in Advanced Pancreatic Cancer under Personalized Peptide Vaccine

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Juzentaihoto (JTT) is a well-known Japanese herbal medicine, which has been reported to modulate immune responses and enhance antitumor immunity in animal models. However, it is not clear whether JTT has similar effects on humans. In particular, there is little information on the effects of JTT in antigen-specific immunity in cancer patients. Here we conducted a randomized clinical study to investigate whether combined usage of JTT could affect antigen-specific immunity and clinical findings in advanced pancreatic cancer patients undergoing personalized peptide vaccination (PPV), in which HLA-matched vaccine antigens were selected based on the preexisting host immunity. Fifty-seven patients were randomly assigned to receive PPV with (n = 28) or without (n = 29) JTT. Unexpectedly, JTT did not significantly affect cellular or humoral immune responses specific to the vaccine antigens, which were determined by antigen-specific interferon-γ secretion in T cells and antigen-specific IgG titers in plasma, respectively. Nevertheless, JTT prevented deterioration of patients' conditions, such as anemia, lymphopenia, hypoalbuminemia, plasma IL-6 elevation, and reduction of performance status, which are frequently observed in advanced cancers. To our knowledge, this is the first clinical study that examined the immunological and clinical effects of JTT in cancer patients undergoing immunotherapy in humans.

1. Introduction

Juzentaihoto (JTT) is a well-known Kampo (Japanese herbal) medicine, which consists of 10 different herbs and has been used as a supplementary therapy in patients with various types of chronic diseases/symptoms, such as fatigue, loss of appetite, night sweats, circulatory problems, and anemia [1]. JTT has also been frequently used for cancer patients, since it was reported to have anti-tumor effects [1–7] and diminish the side effects caused by cancer treatments, such as chemotherapy and radiotherapy [8–12]. In addition, JTT was shown to possess immune-modulating properties, such as enhancement of phagocytosis, cytokine production, antibody production, and NK, NKT, and T-cell functions, in animal experiments [1–7, 13–21]. However, only limited information is available on the immunological and clinical effects of JTT in humans.

Pancreatic cancer, the fourth largest cause of cancer death in the world, is one of the most aggressive cancers [22, 23]. Although there have been substantial advances in the therapeutic modalities for pancreatic cancer, including systemic chemotherapies using gemcitabine (GEM), S-1 (tegaful, gimeracil, and oteracil potassium), and/or molecular-targeted agents, the prognosis of advanced pancreatic cancer patients still remains dismal [22, 23]. Therefore, development

of new therapeutic approaches, including immunotherapy, is needed.

We have developed a novel immunotherapeutic approach, personalized peptide vaccination (PPV), in which HLA-matched peptides were selected and administered, based on the pre-existing host immunity before vaccination [24–28]. Recent clinical trials of PPV have demonstrated feasibility and safety of this new therapeutic approach in various types of advanced cancers [24–28]. For example, in our previous clinical trials, immune responses boosted by vaccination were well associated with overall survival (OS) in advanced pancreatic cancer patients undergoing PPV in combination with GEM as the first-line therapy [28]. In the current study, we conducted a randomized phase II study of PPV to investigate whether combined usage of JTT could show immunological and/or clinical effects in advanced pancreatic cancer patients undergoing PPV.

2. Patients and Methods

2.1. Patients. Patients with pathological and/or clinical diagnosis of pancreatic cancer, who were refractory to conventional treatments, such as surgery, chemotherapy, and radiotherapy, were eligible for inclusion in the current study, if they showed positive IgG responses to at least 2 of the 31 different vaccine candidate peptides, as reported previously [24–28]. Other inclusion criteria were as follows: age of more than 20 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; positive status for the HLA-A2, -A24, -A3 supertype (A3, A11, A31, or A33), or -A26; expected 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; regular use of herbal medicines; pregnancy or nursing; and other inappropriate conditions for enrollment as judged by clinicians. The protocol was approved by the Kurume University Ethical Committee and was registered in the UMIN Clinical Trials Registry (UMIN 000006295). After a full explanation of the protocol, a written informed consent was obtained from all patients before enrollment.

2.2. Clinical Protocol. This was an open-label, randomized phase II study. The patients were randomly assigned to receive PPV with or without oral administration of JTT (PPV plus JTT group versus PPV alone group), according to age and performance status. The primary and secondary objectives were to compare cellular and humoral immune responses to the vaccine antigens and safety between the PPV plus JTT group and the PPV alone group, respectively. Thirty-one peptides, whose safety and immunological effects had been confirmed in previously conducted clinical studies [24–28], were employed for vaccination (12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for HLA-A3 supertype (A3, A11, A31, or A33), and 4 peptides for HLA-A26) (Supplementary Table 1) (see Supplementary Material available online at http://dx.doi.org/10.1155/2013/981717). The peptides were prepared under the conditions of Good Manufacturing Practice (GMP) by PolyPeptide Laboratories (San Diego, CA, USA) and the American Peptide Company (Vista, CA, USA).

The peptides for vaccination to individual patients were selected in consideration of the pre-existing host immunity before vaccination, by assessing the titers of IgG specific to each of the 31 different vaccine candidates, as reported previously [24–28]. A maximum of 4 peptides (3 mg/each peptide), which were selected based on the results of HLA typing and peptide-specific IgG titers, in mixture with incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France), were subcutaneously administered once a week for 6 consecutive weeks. In the PPV plus JTT group, JTT (TJ-48, 15 mg/day; Tsumura Co., Tokyo, Japan) was orally administered for 35 days during the first cycle of 6 vaccinations. After the first cycle of 6 vaccinations, up to 4 vaccine peptides were reselected according to the titers of peptide-specific IgG and administered every 2 weeks. The vaccine peptides were re-selected at every cycle of 6 vaccinations until the discontinuation of PPV. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. Complete blood counts and serum biochemistry tests were performed before and after every cycle of 6 vaccinations.

2.3. Measurement of T-Cell Responses to the Vaccine Peptides. T-cell responses specific to the vaccine peptides were evaluated by interferon (IFN)-γ ELISPOT assay (MBL, Nagoya, Japan). Briefly, peripheral blood mononuclear cells (PBMCs) (2×10^5 cells/well) were cultured in U-bottomed 96-well microculture plates (Nunc, Roskilde, Denmark) with 200 μL of medium (OpTmizer T-Cell Expansion SFM; Invitrogen, Carlsbad, CA, USA) containing 10% FBS (MP Biologicals, Solon, OH, USA), IL-2 (20 IU/mL; AbD Serotec, Kidlington, UK), and each peptide (10 μM). Half of the medium was replaced with new medium containing the corresponding peptides (20 μM) at day 3. After incubation for the following 4 days, the cells were harvested and tested for their ability to produce IFN-γ in response to the corresponding specific peptides. The cells were also tested for IFN-γ production in response to negative control peptides from human immunodeficiency virus (HIV), which might activate nonspecific immune cells, including non-specific CD8 or CD4 T cells and NK cells. IFN-γ secretion after 18-hour incubation was determined by ELISPOT assay with an ELISPOT reader (ImmunoSpot S5 Versa Analyzer; Cellular Technology Ltd., Shaker Heights, OH, USA). All assays were carried out in quadruplicate. The two-tailed Student's t-test was used for statistical evaluation. Antigen-specific T-cell responses were considered positive, when the spot numbers in response to the specific peptides were significantly higher ($P < 0.05$) than those in response to the control HIV peptides, which were supposed to reflect the numbers of immune cells nonspecifically producing IFN-γ. Peptide-specific T-cell responses were shown as the differences between the spot numbers per 1×10^5 PBMCs in response to the specific peptides and those in response to the control peptides.

Table 1: Characteristics of the enrolled patients.

Factor	PPV + JTT (n = 28)	PPV alone (n = 29)	P valuc
Age (years)			0.389
Median (range)	66 (50–83)	65 (45–79)	
Gender			0.922
Male	18	19	
Female	10	10	
Performance status			0.706
0	19	22	
1	9	7	
HLA type			0.753
A24	18	15	
A2	12	13	
A3 supertype	10	17	
A26	5	7	
Clinical stage			0.845
IV	19	20	
Recurrence	9	9	
Location of the main tumor			0.182
Head	6	12	
Body-tail	22	17	
Number of previous chemotherapy regimens			0.843
0	1	1	
1	11	13	
2	13	10	
>3	3	5	
Number of vaccinations			0.443
Median (range)	9 (3–17)	10 (3–18)	
Combination chemotherapy			0.640
None	4	0	
Gemcitabine	10	13	
S-1	5	7	
Gemcitabine + S-1	7	8	
Others	2	1	

2.4. Measurement of Humoral Immune Responses to the Vaccine Peptides. The humoral immune responses specific to the vaccine peptides were determined by peptide-specific IgG titers using a bead-based multiplex assay with the Luminex 200 system (Luminex, Austin, TX, USA), as reported previously [29]. In brief, plasma (×100 diluted) was incubated with 100 μL of peptide-coupled color-coded beads for 1.5 hours at 30°C, followed by washing and incubation with 100 μL of biotinylated goat anti-human IgG (Vector Laboratories, Burlingame, CA, USA) for 1 hour at 30°C. The beads were washed and incubated with 100 μL of streptavidin-PE (Invitrogen) for 30 min at 30°C. After washing, the fluorescence of the beads was detected using the Luminex 200 system. If peptide-specific IgG titers in the postvaccination plasma were more than 2-fold higher than those in the prevaccination plasma, the changes were considered to be significant. If a significant increase was observed in at least one of the vaccine peptides, the antigen-specific humoral immune response was considered to be augmented.

2.5. Measurement of Laboratory Markers. ELISA kits were used to measure serum amyloid A (SAA) (Invitrogen), IL-6 (eBioscience, San Diego, CA, USA), IL-18 (MBL), and C-reactive protein (CRP), IL-12 and TGF-β1 (R&D systems, Minneapolis, MN, USA). Bead-based multiplex assays were used to measure Th1/Th2 cytokines, including IFN-γ, IL-2, IL-4, IL-5, and IL-10 (Human Th1/Th2 5-Plex, Invitrogen), with the Luminex 200 system (Luminex). Frozen plasma samples were thawed, diluted, and assayed in duplicate in accordance with the manufacturer's instructions. The mean of duplicate samples was used for statistical analysis.

Free-radical elective evaluator (Wismerll, Tokyo, Japan) was used to measure biological antioxidant potential (BAP) and derivatives of reactive oxidative metabolites (d-ROM), an index of oxidative stress. Frozen plasma samples were thawed, diluted, and assayed in accordance with the manufacturer's instruction.

2.6. Flow Cytometric Analysis of a Suppressive Immune Cell Subset in PBMCs. A suppressive immune cell subset, myeloid-derived suppressor cells (MDSCs), in PBMCs was examined by flow cytometry. For analysis of MDSCs, PBMCs (0.5×10^6) were incubated for 30 min at 4°C with monoclonal antibodies (mAbs) against lineage markers (CD3, CD14, CD19, and CD56), CD33, and HLA-DR. After washing, the samples were run on a FACSCanto II (BD biosciences, San Diego, CA, USA), and data were analyzed using the Diva software (BD biosciences). All mAbs were purchased from Biolegend (San Diego, CA). Granulocytic MDSCs were identified as CD33 positive in the cell subset negative for both the lineage markers and HLA-DR. Monocytic MDSCs were identified as CD14 positive and HLA-DR negative. The frequency of MDSCs in the mononuclear cell gate defined by the forward scatter and side scatter was calculated.

2.7. Statistical Methods. The Wilcoxon signed-rank test, Student's t-test, the chi-square test, or Fisher's exact test was used to compare differences between measurements. OS was calculated from the first date of peptide vaccination until the date of death or the last date when the patient was known to be alive. Curves for OS were estimated by the Kaplan-Meier method, and the log-rank test was conducted for the comparison of survival curves. Two-sided P values of <0.05 were considered as statistically significant. All statistical analyses were conducted using the JMP version 10.0 software (SAS Institute Inc., Cary, NC, USA).

Table 2: Adverse events.

Adverse events	PPV + JTT (n = 28)				Total (%)	PPV alone (n = 29)				Total (%)
	G1	G2	G3	G4		G1	G2	G3	G4	
Injection site reaction	15				15 (54%)	20				20 (69%)
Blood/bone marrow										
Leukopenia	3	2			5 (18%)	4				4 (14%)
Lymphopenia	3	2			5 (18%)	3	1			4 (14%)
Anemia	3	4			7 (25%)	2	4			6 (21%)
Thrombocytopenia	1	1			2 (7%)	2		1		3 (10%)
Laboratory										
AST increased	2	1			3 (11%)	4	2			6 (21%)
ALT increased	4	1			5 (18%)	3	2			5 (17%)
Bilirubin increased		1			1 (4%)	1				1 (3%)
GGT increased	1	8	1	1	11 (39%)	1	4	3		8 (28%)
ALP increased	2	1	1		4 (14%)	1	2			3 (10%)
Creatinine increased	1				1 (4%)					0 (0%)
Hypoalbuminemia	6	1			7 (25%)	6	2			8 (28%)
Glucose intolerance			1		1 (4%)					0 (0%)
Hyponatremia		1			1 (4%)	2				2 (7%)
Hyperkalemia		1			1 (4%)	1				1 (3%)
Gastrointestinal disorders										
Nausea	1				1 (4%)	1	1			2 (7%)
Diarrhea	2				2 (7%)					0 (0%)
Constipation		1			1 (4%)		1			1 (3%)
Abdominal pain	1				1 (4%)	1	2			3 (10%)
Gastroesophageal reflux disease		1			1 (4%)					0 (0%)
Ascites			1		1 (4%)					0 (0%)
Biliary tract infection			1		1 (4%)		1			1 (3%)
Anorexia		3			3 (11%)	1	1	1		3 (10%)
Fever		1			1 (4%)	3				3 (10%)
Pain		2			2 (7%)	2		1		3 (10%)
Edema limbs	1				1 (4%)		2			2 (7%)
Insomnia					0 (0%)		1			1 (3%)
Rash acneiform					0 (0%)	1				1 (3%)

3. Results

3.1. Patients' Characteristics. Between September 2011 and December 2012, a total of 57 advanced pancreatic cancer patients, who were refractory to conventional treatments, were enrolled in this study. The patients were randomly assigned in a 1:1 ratio to receive PPV with or without oral administration of JTT (PPV plus JTT, n = 28; PPV alone, n = 29). The demographic and baseline disease characteristics of the enrolled patients are given in **Table 1**. There were no significant differences between the two groups in the clinicopathological characteristics, including age, gender, performance status, HLA-type, clinical stage, location of the main tumor, and numbers of previous chemotherapy regimen(s). The median number of vaccinations was 9 (range 3–17) in the PPV plus JTT group and 10 (range 3–18) in the PPV alone group. Five and 2 patients did not complete the first cycle of 6 vaccinations due to disease progression in the PPV plus JTT group and the PPV alone group, respectively. In the PPV plus JTT group, PPV was combined with GEM (n = 10), S-1 (n = 5), GEM and S-1 (n = 7), or other combinations of chemotherapeutic agents (n = 2). Four patients received PPV alone because they could not tolerate chemotherapy. In the PPV alone group, PPV was combined with GEM (n = 13), S-1 (n = 7), GEM and S-1 (n = 8), or other combination of chemotherapeutic agents (n = 1).

3.2. Adverse Events. Adverse events occurring in the patients are listed in **Table 2**. The most frequent adverse event was injection site reactions in both groups. Severe adverse events (grade 3 or grade 4) were as follows: gamma-glutamyl transpeptidase (GGT) increase (n = 2), alkaline phosphatase (ALP) increase (n = 1), glucose intolerance (n = 1), ascites (n = 1), and biliary tract infection (n = 1) in the PPV plus JTT group; GGT increase (n = 3), thrombocytopenia (n = 1), anorexia (n = 1), and pain (n = 1) in the PPV alone group. There were no significant differences in the overall rates of adverse events between the PPV plus JTT group

Table 3: Cellular and humoral immune responses to the vaccine antigens.

	PPV + JTT	PPV alone	P value
Cellular immune responses to the vaccine antigens*			
Before vaccination	2/27 (7.4%)	4/28 (14.3%)	0.669
After vaccination	5/22 (22.7%)	11/26 (42.3%)	0.260
Humoral immune responses to the vaccine antigens†			
Augmented	10/23 (43.5%)	10/27 (37.0%)	0.643

*Antigen-specific T-cell responses were evaluated by IFN-γ ELISPOT assay before and after the first cycle of vaccination.

†Antigen-specific IgG titers in plasma were evaluated before and after the first cycle of vaccination. If peptide-specific IgG titers in the postvaccination plasma were more than 2-fold higher than those in the prevaccination plasma in at least one of the vaccine peptides, the antigen-specific humoral immune response was considered to be augmented.

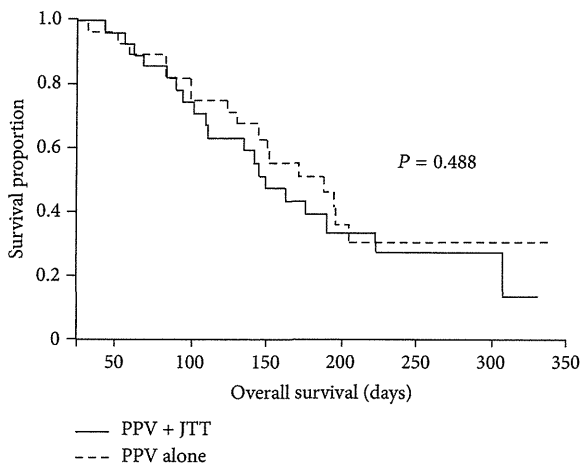


Figure 1: Kaplan-Meier survival analysis in advanced pancreatic cancer patients undergoing PPV with or without JTT. Curves for overall survival were estimated in the PPV plus JTT group (n = 28) and the PPV alone group (n = 29) by the Kaplan-Meier method, and a difference between survival curves was statistically analyzed using the log-rank test.

and the PPV alone group. According to assessment by the independent safety evaluation committee in this trial, all of these severe adverse events were due to cancer progression or other causes, such as side effects related to combined chemotherapies, rather than to the administration of peptide vaccines or JTT.

3.3. Cellular and Humoral Immune Responses to the Vaccine Peptides. Cellular and humoral immune responses specific to the vaccine peptides were analyzed in blood samples before and after the first cycle of vaccination (Supplementary Table 2 and Supplementary Table 3). Since 5 and 2 patients did not complete the first cycle of 6 vaccinations due to disease progression in the PPV plus JTT group and the PPV alone group, respectively, post-vaccination samples of these patients were unavailable.

T-cell responses to the vaccine peptides were measured by IFN-γ ELISPOT assay with PBMCs. PBMCs were available for this assay in 27 and 22 patients before and after the first cycle of vaccination in the PPV plus JTT group, respectively (Supplementary Table 2). In this group, antigen-specific T-cell responses were detectable in 2 of 27 patients (7.4%) and 5 of 22 patients (22.7%) before and after vaccination, respectively. In the PPV alone group, PBMCs were available in 28 and 26 patients before and after the first cycle of vaccination, respectively (Supplementary Table 3). In this group, antigen-specific T-cell responses were detectable in 4 of 28 patients (14.3%) and 11 of 26 patients (42.3%) before and after vaccination, respectively. There were no significant differences between the PPV plus JTT group and the PPV alone group in the antigen-specific T-cell responses both before and after vaccination (P = 0.669 and P = 0.260, resp.) (Table 3).

In addition, the humoral immune responses specific to the vaccine peptides were determined by peptide-specific IgG titers using a bead-based multiplex assay. Plasma samples both before and after the first cycle of vaccination were available in 23 and 27 patients in the PPV plus JTT group and the PPV alone group, respectively (Supplementary Table 2 and Supplementary Table 3). The IgG responses specific to at least one of the vaccine peptides were augmented in 10 of 23 patients (43.5%) and in 10 of 27 patients (37.0%) in the PPV plus JTT group and the PPV alone group, respectively. There was no significant difference in the augmentation of antigen-specific humoral immune responses between the two groups (P = 0.643) (Table 3).

3.4. Clinical Outcome. All the 57 patients were analyzed for OS. Median followup was 148 (95% confidence interval (CI), 123 to 176) days. The median survival times (MST) from the first vaccination were 148 (95% CI, 109 to 222) days and 187 (95% CI, 129 to undefined) days in the PPV plus JTT group and the PPV alone group, respectively. There was no significant difference in OS between groups (P = 0.488, log-rank test) (Figure 1).

In the PPV alone group, 6 of 29 patients showed reduced ECOG performance status during or after the first cycle of vaccination. In contrast, in the PPV plus JTT group, performance status was reduced during or after the first cycle of vaccination in only 3 of 28 patients. A significant change in performance status was observed between before and after (or during) vaccination in the PPV alone group (P = 0.0156, paired Wilcoxon signed-rank test) but not in the PPV plus JTT group (P = 0.125, paired Wilcoxon signed-rank test).

3.5. Laboratory Markers. Laboratory data both before and after the first cycle of vaccination were available in 23 and 27 patients in the PPV plus JTT group and the PPV alone group, respectively. Complete blood counts and serum biochemistry tests were compared between the two groups. There were no significant differences in complete blood counts, such as hemoglobin and lymphocyte counts, and serum biochemistry tests, such as albumin, total bilirubin, and creatinine, before vaccination (Table 4). In the PPV alone group, hemoglobin, lymphocyte counts, and albumin were significantly decreased after the first cycle of vaccination, whereas they did not change significantly after vaccination in the PPV plus JTT group (Figures 2(a), 2(b), and 2(c)). Of note, these results were consistent, even if 4 patients without combined chemotherapies were excluded from the PPV plus JTT group for statistical analysis. This finding suggested that combined usage of JTT prevented the decrease in hemoglobin, lymphocyte counts, and albumin in pancreatic cancer patients undergoing PPV.

In addition, other markers, including cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-18, IFN-γ, and TGF-β1), inflammation markers (CRP and SSA), and oxidative stress markers (d-ROM and BAP), were compared between the PPV plus JTT group and the PPV alone group. There were no significant differences between the two groups in all of these markers examined before vaccination (Table 4). Inflammatory cytokine IL-6 was significantly increased after the first cycle of vaccination in the PPV alone group, but not in the PPV plus JTT group, suggesting that combined usage of JTT inhibited plasma IL-6 elevation in pancreatic cancer patients undergoing PPV (Figure 2(d)). There were no significant changes in other markers between before and after vaccination in the PPV plus JTT group or in the PPV alone group (data not shown). In addition, there were no significant changes in suppressive immune cell subsets, granulocytic and monocytic MDSCs, in PBMCs between before and after vaccination in the PPV plus JTT group or in the PPV alone group (data not shown).

Table 4: Laboratory markers in peripheral blood before vaccination.

Factor	PPV + JTT (n = 28)	PPV alone (n = 29)	P value
Hemoglobin (g/dL)	11.2 ± 1.4*	11.4 ± 1.6	0.4821
Lymphocyte count (/mm^3)	1469.8 ± 482.6	1493.3 ± 409.8	0.8732
Albumin (g/dL)	3.9 ± 0.4	4.1 ± 0.5	0.0895
Creatinine (mg/dL)	1.05 ± 1.90	0.72 ± 0.20	0.6791
Total bilirubin (mg/dL)	0.646 ± 0.473	0.583 ± 0.309	0.7829
IL-2 (pg/mL)	6.17 ± 4.45	4.92 ± 4.42	0.3800
IL-4 (pg/mL)	5.247 ± 15.169	0.662 ± 2.117	0.3160
IL-5 (pg/mL)	0.938 ± 3.887	0.098 ± 0.314	0.8965
IL-6 (pg/mL)	5.037 ± 3.786	4.612 ± 4.089	0.5134
IL-10 (pg/mL)	0.000 ± 0.000	0.062 ± 0.284	0.3415
IL-12 (pg/mL)	0.711 ± 0.793	0.637 ± 0.686	0.5433
IL-18 (pg/mL)	580.9 ± 269.5	571.5 ± 236.6	0.9731
IFN-γ (pg/mL)	2.87 ± 5.48	2.29 ± 6.66	0.4495
TGF-β1 (ng/mL)	5.68 ± 3.08	5.01 ± 1.87	0.7278
C-reactive protein (mg/dL)	1.90 ± 3.50	1.30 ± 1.92	0.2015
Serum amyloid A (μg/mL)	100.66 ± 75.47	69.31 ± 81.49	0.1505
d-ROM (U.CARR)†	267.6 ± 51.4	242.2 ± 86.5	0.2424
BAP‡ (μmol/L)	973.3 ± 261.0	979.3 ± 183.1	0.7442

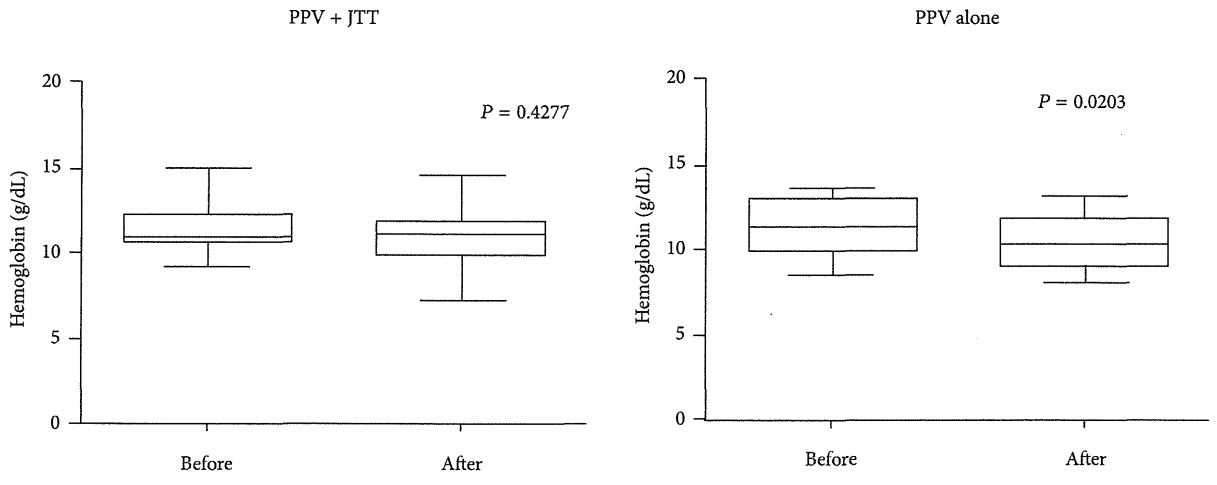
*Values are means ± standard deviations.
†d-ROM: derivatives of reactive oxidative metabolites: U.CARR, Carratelli unit (1 Carratelli unit = 0.8 mg H_2O_2/L).
‡BAP: biological antioxidant potential.

4. Discussion

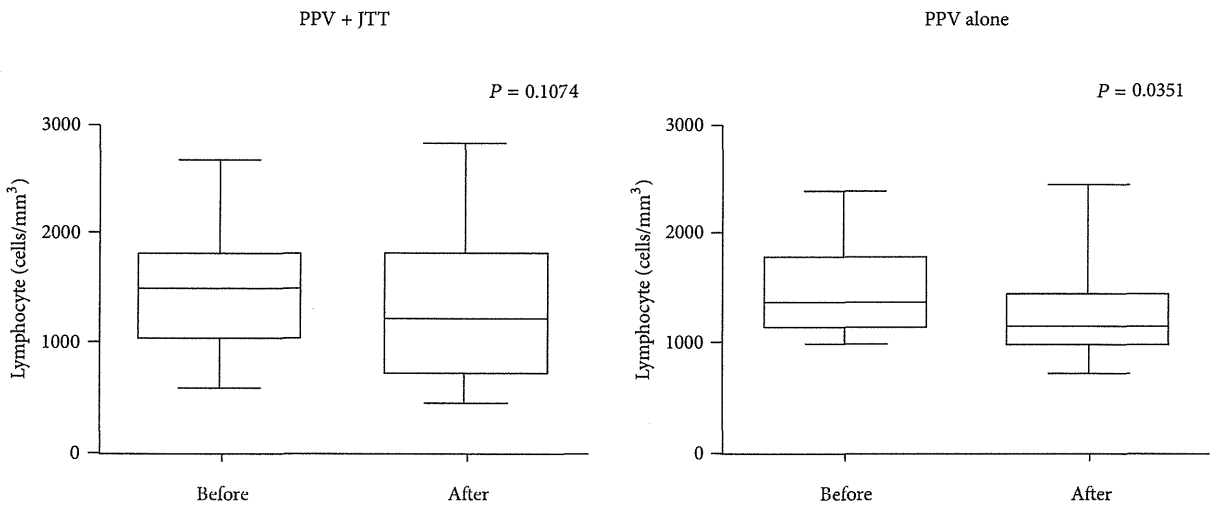
JTT is a well-known Kampo (Japanese herbal) medicine and has been shown to possess immune-modulating and antitumor properties in animal experiments [1–7, 13–21]. However, only limited information is available on the immunological and clinical effects of JTT in cancer patients. To our knowledge, this is the first clinical study that examined the immunological and clinical effects of JTT in cancer patients undergoing immunotherapy in humans.

JTT has been reported to modulate antigen-specific adoptive immune responses in mice [2, 15]. For example, Dai et al. demonstrated that oral administration of JTT induced cytotoxic T cells specific to tumor cells and prevent tumor development in the RET-transgenic mouse model [2]. Iijima et al. reported that JTT induced Th1-skewed immune responses and Th1-dependent antibody responses in aged mice [15]. However, the current study showed that combined usage of JTT did not significantly affect cellular or humoral immune responses to the vaccine antigens after PPV. JTT has also been shown to enhance production of cytokines, such as IL-12 and IL-18, in mice [17, 18]. But, in the current study, there were no significant differences in production of several different cytokines, except IL-6, between the PPV plus JTT group and the PPV alone group. Furthermore, there were no significant differences in suppressive immune cell subsets, granulocytic and monocytic MDSCs [30, 31], in PBMCs between the two groups. Based on our results, combined usage of JTT had no significant immune-modulating effects in advanced cancer patients undergoing PPV, in disagreement with the results of previous animal experiments. In addition, although JTT was reported to inhibit immune cell-mediated oxidative stress [6, 19], the current study showed no significant effects of JTT in redox status, which was determined by oxidative stress markers (d-ROM and BAP) in plasma, in advanced cancer patients undergoing PPV.

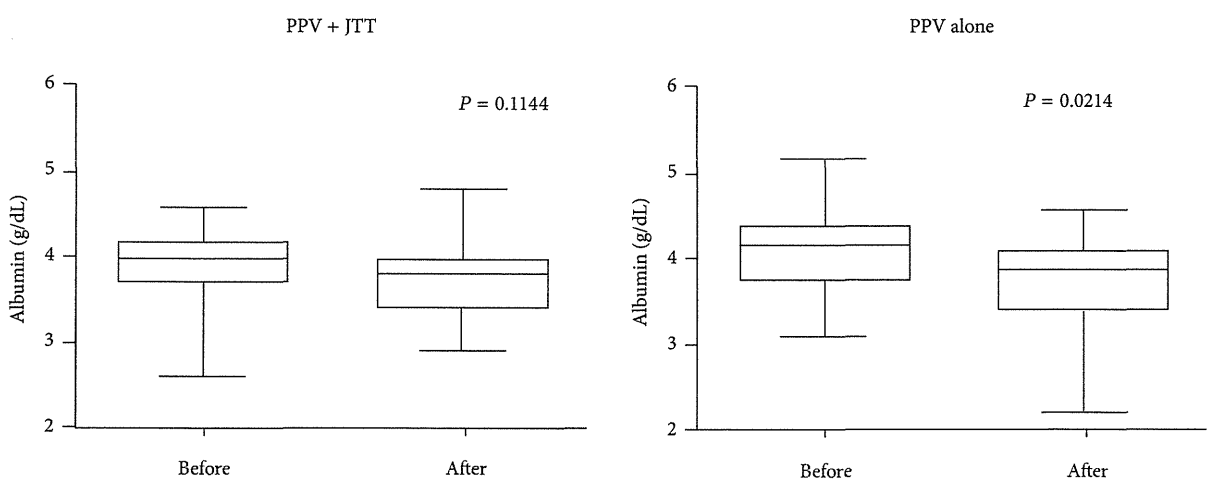
Several previous reports demonstrated that JTT showed antitumor effects through various mechanisms [1–7]. Ohnishi et al. showed that oral administration of JTT before tumor inoculation resulted in dose-dependent inhibition of liver metastasis of colon 26-L5 carcinoma cells [5]. Matsuda et al. also reported that oral administration of JTT before tumor cell injection significantly inhibited lung metastasis



(a)



(b)



(c)

FIGURE 2: Continued.

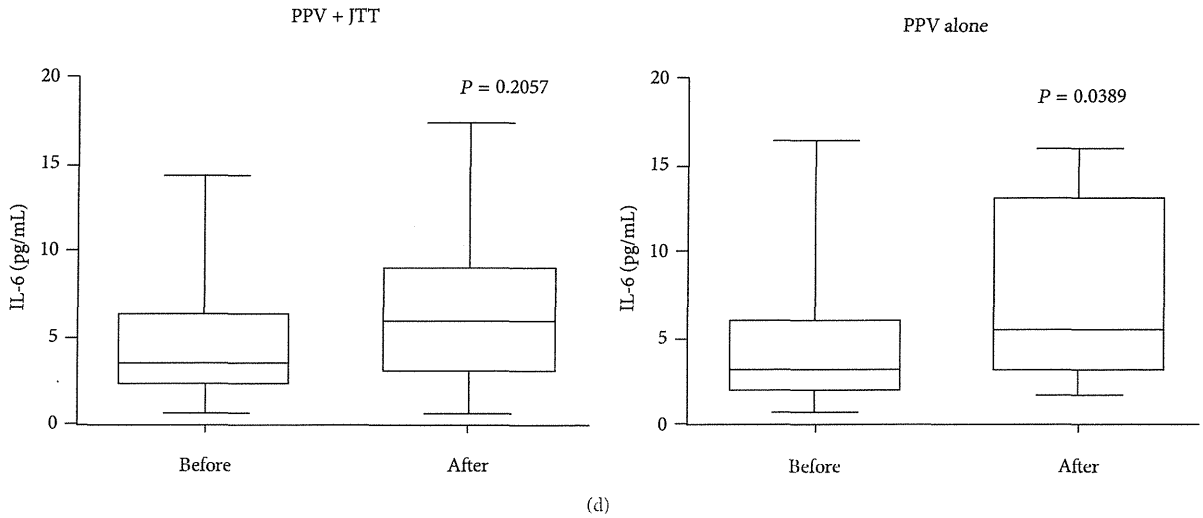


FIGURE 2: Laboratory markers before and after vaccination in advanced pancreatic cancer patients undergoing PPV with or without JTT. Laboratory markers were compared between before and after the first cycle of 6 vaccinations in the PPV plus JTT group (n = 23) and the PPV alone group (n = 27) by the paired Wilcoxon signed-rank test. The levels of hemoglobin (a), lymphocyte counts (b), albumin (c), and IL-6 (d) in peripheral blood before and after vaccination are shown. The results are represented by box-and-whiskers graphs. The box plots show median and interquartile range. The whiskers go down to the lowest value and up to the highest value.

of B16 melanoma cells in mice [4]. In addition, in humans, JTT supplementation was shown to result in considerable improvement in intrahepatic recurrence-free survival in hepatocellular carcinoma (HCC) patients after surgical treatment [6]. Although these results suggested the preventive effects of JTT in tumor development in mice and humans, the therapeutic effects of this agent for advanced stage of tumors are not well defined. The current study showed that combined usage of JTT conferred no survival benefits in patients with pancreatic cancer undergoing PPV.

Combined usage of PPV and JTT was well tolerated. The most frequent adverse event was injection site reactions, and all of the severe adverse events observed were due to cancer progression or other causes rather than to the vaccinations or JTT administration. Of note, JTT administration induced some beneficial effects in pancreatic cancer patients undergoing PPV. Although the patients treated with PPV alone showed decrease in hemoglobin, lymphocyte counts, and albumin after vaccination possibly due to side effects of combined chemotherapies and/or malnutrition mediated by disease progression, those treated with PPV in combination with JTT maintained a stable level of these factors, as previously suggested [1, 12, 32]. Consistent with these findings, a significant change in performance status was observed between before and after (or during) vaccination in the PPV alone group but not in the PPV plus JTT group. These results suggest that JTT has the potential to prevent deterioration of patients' conditions without severe adverse events even in advanced cancer patients undergoing immunotherapy. Other clinical data, such as patients' quality of life (QOL), were unavailable in this study, but they might be worthy of assessment in future clinical trials.

It should also be noted that the elevation of the pro-inflammatory cytokine IL-6 was inhibited by combined usage of JTT. IL-6 is a multifunctional cytokine that regulates various aspects of immune responses, acute phase reactions, and hematopoiesis. In particular, IL-6 has been reported to be deeply involved in inflammation associated with cancer development and progression [33, 34]. Indeed, there have been many studies describing the correlation between IL-6 elevation and poor prognosis in various types of cancers, including pancreas cancer [35–38]. In addition, IL-6 has recently been reported to be one of the critical cytokines for inducing suppressive immune cell subsets, such as MDSCs and Th17, which are known to negatively affect anti-tumor immunity [39–41]. Therefore, the inhibitory effect of JTT on IL-6 elevation might be beneficial for controlling cancer progression.

5. Conclusion

In summary, we for the first time examined the immunological and clinical effects of JTT in cancer patients undergoing cancer vaccination in humans. Our randomized clinical trial of PPV with or without JTT suggested that combined usage of JTT revealed a potential to prevent deterioration of patients' conditions but had no effects in antigen-specific immunity in advanced pancreatic cancer patients. Since all of the enrolled patients had rapidly progressive advanced tumors, it might be possible that JTT supplementation for a limited, short period was not sufficient to elicit beneficial immune responses in the treated patients. A next step of randomized clinical trials of PPV with or without JTT would thus be recommended in

cancer patients in the adjuvant setting or in those with more slowly growing tumors.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Short Communication Open Access

Controversies in Clinical Trials of Cancer Vaccines for Glioblastoma

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Keywords: Glioblastoma; Clinical trial; Tumour progression; Best recommended treatment

Glioblastoma multiforme (GBM), the most common primary brain tumour, continues to have a dismal prognosis. The standard initial treatment for GBM is surgical resection along with postoperative adjuvant therapy, including temozolomide, concomitant with 60 Gy of radiation therapy (RT) [1]. However, most patients eventually relapse and long-term survival remains elusive [2,3]. Thus, novel therapeutic modalities for GBM are being explored, and different types of immune-mediated approaches have been preclinically and clinically evaluated in phase I and II trials [4]. However, these GBM clinical trials face significant limitations in terms of their assessment of tumour progression and protocol setting. A critical and comprehensive review of how GBM trials should be conducted is required with a focus on how progression can be defined and clinical benefits can be evaluated following the administration of cancer vaccines.

Limitations of the Conventional Tumour Progression Criteria

In current clinical trials of therapies for solid Tumours, cessation of treatment is recommended once "progressive disease" (PD) is detected according to the WHO or response evaluation criteria in solid Tumours (RECIST) criteria. In the WHO criteria, PD is defined as at least a 25% increase in the sum of the products of the two largest perpendicular diameters (SPD) compared with nadir and/or unequivocal progression of non-index lesions and/or the appearance of new lesions [5]. In the RECIST criteria, a 20% increase is defined as PD [6]. Criteria developed by Macdonald and colleagues in 1990 have also been used for assessing the anti-Tumour responses of gliomas [7]. These criteria are based on the two-dimensional WHO response criteria and mark the transition from a subjective interpretation of clinical and radiologic changes to a more objective evaluation. Other factors, such as the use of steroids and changes in neurologic status, are also included in the response assessment. Although they are widely accepted, a number of groups have reported a few limitations of these criteria [8-10]. Clinical evidences indicate that the traditional Macdonald's criteria may not be sufficient for completely characterizing responses in the new era of targeted therapies. Thus, ideal progression criteria that can comprehensively describe all patterns of anti-Tumour responses to cancer vaccines for gliomas remain to be developed.

New systematic criteria designated "immune-related response criteria" for describing additional response patterns observed with immunotherapies that cannot be assessed by the traditional RECIST or WHO criteria have recently been defined [11]. In these new criteria, progression is defined as ≥ 25% increase in Tumour burden compared with nadir at two consecutive time points at least 4 weeks apart in the absence of rapid clinical deterioration. However, these novel criteria may also be of limited value for assessing the anti-Tumour responses of gliomas, as explained below.

Tumour Size Threshold for Defining PD

Tumours with enhancement are defined as PD when the changes in the enhancing areas reach 25% according to Macdonald's criteria. However, whether it is appropriate to define a ≥ 25% increase in Tumour size as "PD" remains unknown. In fact, this issue was raised by our retrospective analysis of the personalized peptide ITK-1 vaccine trial for recurrent GBM, where a 54% increase according to the WHO criteria or a 43% increase according to the RECIST criteria was predictive of a high mortality with a sensitivity of 69% (95% confidence interval: 42%-87%) and 85% (58%-96%), respectively (Figures 1A and 1B). Our experience suggests that the Tumour size threshold for defining PD when evaluating the efficacy of cancer vaccines remains to be carefully determined.

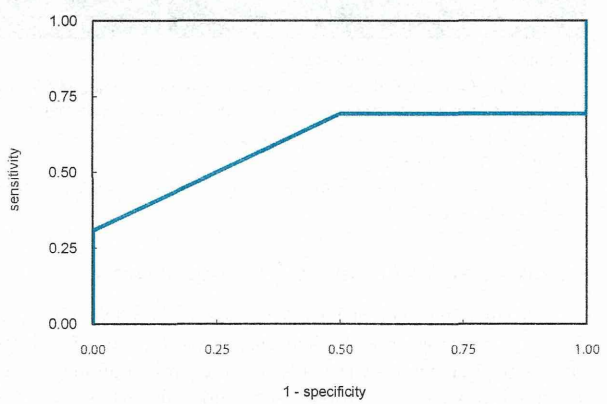

Figure 1a: ROC curve of increasing rate of tumor burden predicting mortality at progression according to the WHO criteria. The area under the ROC curve was 0.69 (95% CI: 0.43, 0.95). Increase in the SPD of at least 54% is predictive of mortality at progression with a sensitivity of 67% (42%, 87%) and a specificity of 50% (9%, 91%).

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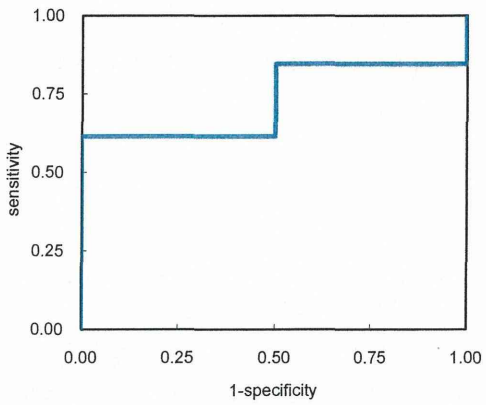

Figure 1b: ROC curve of increasing rate of tumor burden predicting mortality at progression according to the RECIST criteria. The area under the ROC curve was 0.73 (95% CI: 0.42, 1.00). Increases in the largest perpendicular diameters of at least 43% are predictive of mortality at progression with a sensitivity of 85% (58%, 96%) and a specificity of 50% (9%, 91%).

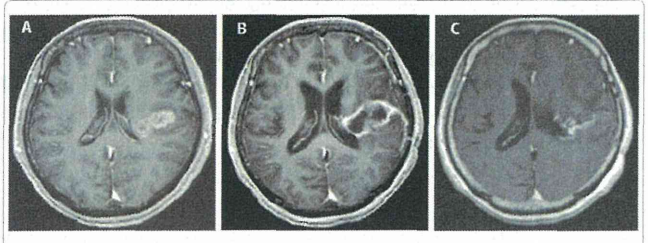

Figure 2: Example of pseudoprogression after vaccination.

(A) T1-weighted contrast-enhanced magnetic resonance image (MRI) from a 59-year-old patient with biopsy-proven glioblastoma before vaccination.

(B) Eight weeks after vaccination, a significant increase in contrast enhancement was shown.

(C) On a follow-up MRI 24 weeks later, a significant reduction was observed in the enhancing lesions.

Controversy Evaluating Enhancing Lesions

Tumour enhancement has been assessed based on the extent of Tumour-occupying lesions when evaluating Tumour size. However, considering that clinical trials of cancer vaccines for gliomas have been attempted in patients at various stages and with various conditions of disease, tumour enhancement may be influenced by not only cancer cell occupation but also by several other factors, including postsurgical changes, disruption of the blood-brain barrier, inflammation, radiation necrosis, and use of corticosteroids [12-17]. These changes in enhancing areas are not always directly correlated with those of Tumour-occupying lesions. Stable disease (SD) in the enhancing areas might be considered an indicator of significant therapeutic effects in cancer vaccine trials [18]. For example, it is possible that enhancement within Tumours may be, at least in part, attributed to autoimmune responses and/or brain inflammation caused by systemic immunization [4].

Tumour Regression after Apparent PD

Clinical studies of cancer vaccines have in certain cases shown that initial induction of SD or PD is followed by subsequent Tumour regression, raising concerns about evaluation of anti-Tumour responses using the WHO or RECIST criteria [11,19]. Such radiological increases in Tumour volumes that precede beneficial clinical responses in patients administered cancer vaccines may be attributed to either continued Tumour growth until sufficient anti-Tumour activity develops, or to transient infiltration of immune cells. In addition, transient increases in enhancement without actual Tumour progression, known as "pseudo progression", have been reported in multiple studies of immunotherapeutic agents [20,21]. For example, in our previous cancer vaccine trial, significant clinical effects after 12 weeks, and in certain cases even after 24 weeks, were observed in a subset of patients with apparent PD according to the classical progression criteria (Figures 2A, 2B and 2C) [22]. Considering the fact that follow-up observations cannot be mandated in patients with PD in most clinical trial protocols, the actual number of patients with beneficial clinical responses after PD may be underestimated. This could limit the value of progression-free survival as a primary end point in cancer vaccine trials.

Collectively, clinical development of cancer vaccines has been hampered by the absence of ideal progression criteria that can comprehensively describe all patterns of anti-Tumour response. Establishment of specific guidelines for classifying Tumour progression to evaluate anti-Tumour activities remains an urgent issue in relation to cancer vaccine trials for gliomas.

Overall Survival as a Primary Endpoint in Cancer Vaccine Trials for Gliomas

Since the numbers of patients with high-grade glioma, particularly GBM, are limited, it would be quite difficult to conduct large-scale immunotherapy trials for this disease [4,12]. The number of patients receiving treatments is relatively small in cancer vaccine trials, and the evaluation criteria vary depending on the trial [22-42]. Such large variations in immune-based therapeutic approaches for GBM make direct comparison difficult. Given this situation, the immunotherapy field needs to urgently address what clinical benefits can be detected in such small-scale, limited clinical trials, and how these can be evaluated. One possibility would be to concentrate on evaluating overall survival (OS). Because of a lack of effective treatments for refractory GBM, the effect of a particular treatment on OS may not be influenced by subsequent salvage treatments.

Combination with the Best Recommended Treatment

A novel hypothetical consideration may be combination therapy with additional agents in GBM vaccine trials, which may enhance the clinical effects of cancer vaccines. Recently, concomitant treatments including RT, chemotherapies, and targeted therapies, have been reported to enhance the therapeutic effects of cancer vaccines through multiple immune-related mechanisms (i.e., activation of antigen-presenting cells or cytotoxic T cells and removal of suppressor cells) [43,44]. Several clinical studies have shown that chemotherapies combined with cancer vaccines can have a synergistic effect [44]. Synergistic effects of salvage chemotherapies after therapeutic cancer vaccination were also reported to improve patient survival in two clinical studies of GBM and small cell lung cancer [45,46]. Sampson et al. [47] reported that cancer vaccination after concomitant RT and temozolomide provided a survival advantage of 9 months compared with control patients in a phase II multicenter trial in patients with newly diagnosed GBM. These clinical studies illustrate that cancer vaccines combined with other treatment modalities may provide a valid therapeutic option for GBM. Therefore, the best recommended treatment (BRT) could be combined with chemotherapies and/or radiotherapies but not with best supportive care (BSC) in clinical trials of cancer vaccines for GBM. This will facilitate the occurrence

of synergistic effects, although the appropriate doses and schedules for optimal synergy between chemotherapies and cancer vaccines remain to be determined.

Considering the disease rarity and the limited survival benefit derived from cancer vaccines for GBM, the employment of BRT (but not of BSC), which could synergistically enhance the clinical effects of the cancer vaccines, would be a breakthrough for accelerated development of cancer vaccines. The FDA also supports this type of combination therapy in their guidelines for the development of therapeutic cancer vaccines [48].

Disclosure Statement

No part of this report has been previously presented elsewhere.

Terasaki received a research grant from the Suzuken Memorial Foundation, Itoh have stock ownership in the Green Peptide Co., Nishikawa had honoraria from the MSD Co, Ltd.

Conflict of Interest

The authors report no potential conflict of interest except for Itoh.

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