clinical trial of PPV and another 19 who were not receiving PPV therapy. Paraffin-embedded tissue samples were cut into 4-μm sections and labeled on a BenchMark XT (Ventana Medical Systems, Tucson, AZ) with antibodies to the tumor antigens. The DAB (Ventana iVIEW DAB Detection Kit; Ventana Medical Systems) was used for the detection of antigens.

Patients

Patients with histological diagnosis of ovarian, fallopian tubal or primary peritoneal cancer were eligible for inclusion in this study. They also had to show positive IgG responses to at least two of the HLA-class I-matched vaccine candidate peptides. The other inclusion criteria as well as exclusion criteria were not largely different from those of other previously reported clinical studies[17–20]: an age between 20 and 80 years; an Eastern Cooperative Oncology Group performance status (PS) of 0 or 1; positive status for HLA-A2, -A3, -A11, -A24, -A26, -A31, or -A33; life expectancy of at least 12 weeks; and adequate hematologic, renal and hepatic function (>2500/μL of white blood cells, ≥1000/μL of lymphocytes, >80 000/μL of platelets, <1.5 mg/dL of serum creatinine and <2.5 mg/dL of total bilirubin). Patients with lymphocyte counts of <1000 cells/μL were excluded from the study, since we previously reported that pre-vaccination lymphopenia is an unfavorable factor for OS in cancer patients receiving PPV[21,22]. Other 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 the clinicians. The protocols were approved by the Kurume University Ethical Committee and were registered in the UMIN Clinical Trials Registry (UMIN#3083 for 40 patients and UMIN#1482 for 2 patients). After a full explanation of the protocol, written informed consent was obtained from all patients before enrollment.

Clinical protocol

The aim of this study was to investigate the feasibility of PPV as a therapeutic cancer vaccine from the viewpoint of OS of recurrent ovarian cancer patients, along with prognostic factors for OS, safety and immunological response in ovarian cancer patients under PPV. Thirty-one peptides, whose safety and immunological effects had been confirmed in previously conducted clinical studies[17–20], were employed for vaccination (Table 1). The peptides were prepared under the conditions of Good Manufacturing Practice by the PolyPeptide Laboratories (San Diego, CA) and American Peptide Company (Vista, CA). The appropriate peptides for vaccination in individual patients were selected in consideration of the HLA-type and pre-existing host immunity before vaccination, as assessed by IgG levels against each of the 31 different vaccine candidates as described previously[23]. Similarly, the concomitant chemotherapy was permitted during the vaccination for patients who could tolerate it. A maximum of four peptides (3 mg/each peptide) were subcutaneously administrated with Montanide ISA51VG (Seppic, Paris, France) once a week for six consecutive

Table 1. Vaccine candidate peptides used for PPV.

Peptide name	Original protein	Position	Sequence	HLA-IA restriction	References
CypB-129	Cyclophilin B	129–138	KLKHYGPGWV	A2/A3 supertype	Jpn J Cancer Res 2001;92:762–767.
Lck-246	p56^{lck}	246–254	KLVERLGAA	A2	Int J Cancer 2001;94:237–242.
Lck-422	p56^{lck}	422–430	DVWSFGILL	A2/A3 supertype	Int J Cancer 2001;94:237–242.
ppMAPkkk-432	ppMAPkkk	432–440	DLLSHAFFA	A2/A26	Cancer Res 2001;61:2038–2046.
WHSC2-103	WHSC2	103–111	ASLDSDPWV	A2/A26/A3 supertype	Cancer Res 2001;61:2038–2046.
HNRPL-501	HNRPL	501–510	NVLHFFNAPL	A2/A26	Cancer Res 2001;61:2038–2046.
UBE2V-43	UBE2V	43–51	RLQEWCSVI	A2	Cancer Res 2001;61:2038–2046.
UBE2V-85	UBE2V	85–93	LIADFLSGL	A2	Cancer Res 2001;61:2038–2046.
WHSC2-141	WHSC2	141–149	ILGELREKV	A2	Cancer Res 2001;61:2038–2046.
HNRPL-140	HNRPL	140–148	ALVEFEDVL	A2	Cancer Res 2001;61:2038–2046.
SART3-302	SART3	302–310	LLQAEAPRL	A2	Int J Cancer 2000;88:633–639.
SART3-309	SART3	309–317	RLAEYQAYI	A2	Int J Cancer 2000;88:633–639.
SART2-93	SART2	93–101	DYSARWNEI	A24	J Immunol 2000;164:2565–2574.
SART3-109	SART3	109–118	VYDYNCHVDL	A24/A24/A3 supertype	Cancer Res 1999;59:4056–4063.
Lck-208	p56^{lck}	208–216	HYTNASDGL	A24	Eur J Immunol 2001;31:323–332.
PAP-213	PAP	213–221	LYCESVHNF	A24	J Urol 2001;166:1508–1513.
PSA-248	PSA	248–257	HYRKWIKDTI	A24	Prostate 2003;57:152–159.
EGF-R-800	EGF-R	800–809	DYVREHKDNI	A24	Eur J Cancer 2004;40:1776–1786.
MRP3-503	MRP3	503–511	LYAWEPSFL	A24	Cancer Res 2001;61:6459–6466.
MRP3-1293	MRP3	1293–1302	NYSVRYRPGL	A24	Cancer Res 2001;61:6459–6466.
SART2-161	SART2	161–169	AYDFLYNYL	A24	J Immunol 2000;164:2565–2574.
Lck-486	p56^{lck}	486–494	TFDYLRSVL	A24	Eur J Immunol 2001;31:323–332.
Lck-488	p56^{lck}	488–497	DYLRSVLEDF	A24	Eur J Immunol 2001;31:323–332.
PSMA-624	PSMA	624–632	TYSVSFDSL	A24	Cancer Sci 2003;94:622–627.
EZH2-735	EZH2	735–743	KYVGIEREM	A24	Prostate 2004;60:273–281.
PTHrP-102	PTHrP	102–111	RYLTQETNKV	A24	Br J Cancer 2004:287–296.
SART3-511	SART3	511–519	WLEYYNLER	A3 supertype	Cancer Immunol Immunother 2007;56:689–698.
SART3-734	SART3	734–742	QIRPIFSNR	A3 supertype	Cancer Immunol Immunother 2007;56:689–698.
Lck-90	p56^{lck}	90–99	ILEQSGEWWK	A3 supertype	Br J Cancer 2007;97:1648–1654.
Lck-449	p56^{lck}	449–458	VIQNLERGYR	A3 supertype	Br J Cancer 2007;97:1648–1654.
PAP-248	PAP	248–257	GIHKQKEKSR	A3 supertype	Clin Cancer Res 2005;11:6933–6943.

weeks. After the first cycle of six vaccinations, peptides were administered every two weeks. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0 (CTCAE Ver4, Bethesda, MD). Complete blood counts and serum biochemistry tests were performed at every sixth vaccination. The clinical responses were evaluated using the Response Evaluation Criteria in Solid Tumors in the vaccinated patients, whose radiological findings by computed tomography scan or magnetic resonance imaging were available before and after vaccinations.

Measurement of humoral and cellular responses

The IgG levels to each of the 31 peptide candidates were measured using the Luminex system (Luminex, Austin, TX), as previously reported[23]. If the titers of peptide-specific IgG to at least one of the vaccinated peptides in the post-vaccination plasma were more than twofold higher than those in the pre-vaccination plasma, the changes were considered to be significant. In addition, if the numbers of HLA-A-matched peptides reactive to peptide-specific IgG increased or decreased at the sixth vaccination, this was considered epitope spreading (ES) or epitope decline (ED), respectively.

Cellular responses were evaluated by INF-γ ELISPOT assay as previously described[23]. Antigen-specific T cell responses were evaluated by the difference between the numbers of spots produced in response to each corresponding peptide and that produced in response to the control HIV peptide; a difference of at least 30 spots per 10^5 PBMCs was considered positive or detectable and the subtracted spot numbers are shown. In negative cases, spot numbers are shown as "zero". If the post-vaccination values were more than twofold higher than the pre-vaccination values, this was considered an augmented response. If the pre-vaccination values were "zero", then post-vaccination values of more than 30 were considered an augmented response.

Flow-cytometric analysis of PBMCs

For the analysis of MDSCs, PBMCs were stained with the following antibodies as previously described[24]: anti-CD3-FITC, anti-CD56-FITC, anti-CD19-FITC, anti-CD33-APC, anti-HLA-DR-PE/Cy7 and anti-CD14-APC/Cy7 antibodies. In the cell subset negative for the lineage markers (CD3, CD19, CD56 and CD14) and HLA-DR, MDSCs were identified as $CD33^+$. The samples were analyzed on a FACSCanto II with Diva software (BD Biosciences, San Diego, CA). Antibodies were purchased from Biolegend (San Diego, CA) and BD Biosciences.

Statistical analysis

A two-sided Wilcoxon test was used to compare differences between pre- and post-vaccination measurements. p Values <0.05 were considered statistically significant. OS time was calculated from the first day of peptide vaccination until the date of death or the last date when the patient was known to be alive. Predictive factors for OS were evaluated by univariate analysis with the Cox proportional hazards regression model. All statistical analyses were conducted using the JMP version 8 or SAS version 9.1 software (SAS Institute Inc., Cary, NC).

Results

General tumor expression of parental proteins of vaccine peptides

To confirm the general expression of the 15 different parental TAAs of the vaccine candidate peptides shown in Table 1, tumor specimens from 22 ovarian cancer patients, including three patients (FOV-019, -028 and -030) who were enrolled in a clinical trial of PPV and 19 patients who were not being treated with PPV, were subjected to immunohistochemical analysis. The results showed that 13 TAAs were detectable in the ovarian cancer cells tested. Nine of them were expressed in the majority of cancer cells tested, whereas MRP3, EGF receptor, PAP and lck were expressed in only a portion of the cancer cells. Representative staining patterns are shown in Figure 1. In contrast, the two prostate-related vaccine antigens (PSMA and PSA) were not detectable in any tissues tested, as expected from the previous studies listed in Table 1.

Patient characteristics

Between January 2009 and December 2012, 37 patients with epithelial ovarian cancer, three with fallopian tube cancer and two with primary peritoneal cancer were enrolled in this study. All patients had recurrence and persistence of disease. The characteristics of the 42 patients are listed in Table 2. Serous adenocarcinoma was the most common histology (52.2%). Seventeen patients had platinum-sensitive and 25 had platinum-resistant recurrence. All patients had achieved a documented response to initial platinum-based treatment and had been off therapy until recurrence. Platinum sensitivity or resistance was defined as an off therapy period of longer or shorter than six months after initial platinum-based treatment, respectively. Before enrollment, all the patients underwent additional chemotherapy against recurrent tumor. The median duration from the first recurrence to the PPV was 14.5 months, ranging from 1 to 89. PS at the time of enrollment was grade 0 ($n=33$) or grade 1 ($n=9$). During the PPV, 22 patients underwent concomitant chemotherapy, and the remaining 20 patients did not tolerate concomitant chemotherapy (Table 2).

Toxicities

Grade 1 or 2 dermatological reaction at the injection sites was observed in all cases (Table 3). The high grade adverse events (more than grade 3) were anemia (grade 3: $n=2$; grade 4: $n=1$), leukocytopenia (grade 4: $n=1$), neutropenia (grade 3: $n=2$; grade 4: $n=1$), lymphopenia (grade 3: $n=1$), hypoalbuminemia (grade 3: $n=1$) and infection of the injection site (grade 3: $n=1$). Except for infection of the injection site, all of these severe adverse events were concluded to be associated with chemotherapy, rather than directly associated with the vaccinations, based on the assessment of an independent safety evaluation committee. However, infection of the injection site (a lower limb) was concluded to be a vaccination-related adverse event.

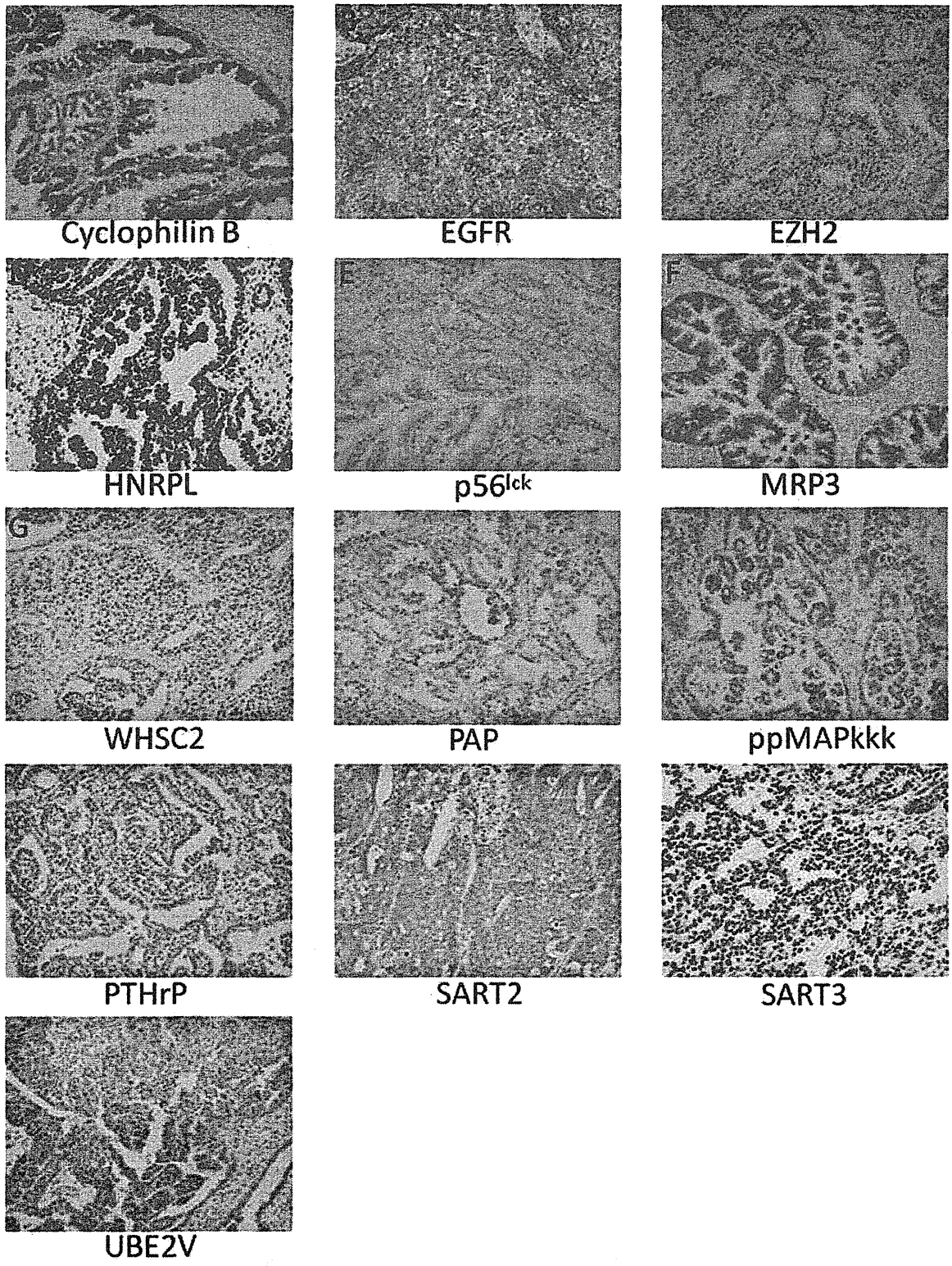

Figure 1. Expression profile of parental proteins of vaccine peptides. Tumor specimens from 22 ovarian cancer patients, including three patients who were enrolled in a clinical trial of PPV and 19 patients who were not being treated with PPV, were subjected to immunohistochemical analysis.

Table 2. Characteristics of the enrolled patient with recurrent ovarian cancer ($n=42$).

Parameters	n
Age	
median (range)	57.5 (22–80)
Origin	
Ovary	37
Fallopian tube	3
Periosteum	2
Histology	
Serous	22
Endometrioid	7
Mucinous	3
Clear	3
Others	7
HLA	
A2	10
A24	30
A3 superfamily	26
A26	5
Performance status	
0	33
1	9
Number of prior regimen	
1	4
2	10
3	14
≥4	14
Platinum sensitivity	
Sensitive	17
Resistant	25
Combined chemotherapy	
Yes	22
No	20

Table 3. Toxicities.

	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Injection site reaction	0	10	32		
Blood/Bone marrow					
Anemia	23	6	10	2	1
Leukocytopenia	32	8	1		1
Neutropenia	38	1		2	1
Lymphopenia	24	13	3	2	
Thrombocytopenia	39	1	2		
Laboratory					
AST elevation	37	5			
ALT elevation	39	3			
Hypoalbuminemia	20	16	5	1	
Creatinine elevation	33	9			
Renal/genitourinary					
Obstruction: ureter	41		1		
Intestine					
Intestinal bleeding	41			1	
Pain					
Tumor	40		2		
Leg edema	40	2			
Infection					
Injection site	41			1	

Immune responses to the vaccinated peptides

Both humoral and cellular immune responses specific for the peptides used for vaccination were analyzed in blood samples of the patients collected at pre-vaccination and at the 6th and 12th vaccinations (Table 4). Due to disease progression, 12 patients failed to complete the second cycle of vaccinations (12th vaccination), while one patient decided to withdraw from the study before the 12th vaccination. Peptide-specific IgGs reactive to each of 31 different peptides, including both vaccinated and non-vaccinated peptides, were measured.

Augmentation of the IgG responses specific for at least one of the vaccinated peptides was observed in 16 of 42 recurrent cases at the time of the 6th vaccinations. The 12th vaccination induced the augmentation in 29 of 30 recurrent cases tested. In addition, the numbers of HLA-A-matched peptides reactive to peptide-specific IgG increased in 16 cases, whereas it decreased in the other 16 cases at the 6th vaccination. In this study, the former phenomenon was referred to as ES and the latter phenomenon as ED.

CTL responses to the vaccinated peptides were measured by IFN-γ ELISPOT assay. Representative well images of ELISPOT assay are shown in Figure 2. Antigen-specific CTL responses were detectable in only 12 of 42 patients before vaccination. Augmentation of CTL responses specific for at least one of the vaccinated peptides was observed in 18 of 42 and 19 of 30 cases at the time of the 6th and 12th vaccinations, respectively. Interestingly, ES was well correlated with the augmentation of IgG and CTL responses ($p=0.014$, $p=0.044$), but no correlation was observed between the augmentation of IgG and CTL responses ($p=0.101$).

Cytokines and inflammation markers

Significant increases of IL-6, CRP and SAA levels were observed after the sixth vaccination ($p=0.0012$, $p=0.001$ and $p=0.010$, respectively) (Figure 3A). Furthermore, plasma CRP levels before vaccination were higher in the group that showed an augmentation of IgG response at the sixth vaccination ($p=0.031$).

Flow-cytometric analysis of PBMCs

Immune cell subsets in both pre- and sixth vaccination PBMCs were examined by flow cytometry. No significant difference was found between the frequencies of pre- and sixth vaccination of MDSC and $CD3^+CD26^+$ cells. The median frequency of MDSC in pre-vaccination PBMCs was lower in the group that showed an augmentation of CTL response after vaccination ($p=0.005$, Student's t-test) (Figure 3B). The frequencies of $TNFRSF14^+$ cells in both the $CD11^+$ and $CD11^-$ subsets were not changed between before and after vaccination (data not shown). However, the frequency of $CD11^+TNFRSF14^+$ before vaccination was higher in the group that showed an augmentation of IgG response after vaccination ($p=0.019$, Student's t-test).

Relationship between clinical findings or immunological responses and OS

The median number of vaccinations was 12, with a range of 6 to 33. Among the 25 vaccinated patients whose radiological findings were available both before and after the vaccination, 1 patient (FOV-027) had a complete response (CR), and this patient was treated with a combination of PPV

Table 4. Antigen expression, immunological responses and clinical outcome of each patient.

ID (HLA-A type)	Peptides for vaccination	Antigen expression in tumor tissue	IgG response (FIU)			CTL response (IFN-γ producing cells/10^5 cells)			Numbers of IgG positive peptides			Best clinical response	Overall survival (months)	Prognosis
			Pre	6th	12th	Pre	6th	12th	pre	6th	12th			
F-018 (A2/A24)	ppMAPkkk-432	na	1492	1310	na	0	0	na	6	5	na	PD	19.1	DOD
	WHSC2-103		1875	1904	na	0	0	na						
	HNRPL-140		897	840	na	0	0	na						
	MRP3-503		230	149	na	0	0	na						
F-040 (A11/A24)	PAP-213	na	3501	2667	na	0	371	na	7	8	na	PD	48.5	DOD
	MRP3-503		173	59	na	0	774	na						
	SART3-511		108	79	na	0	268	na						
	WHSC2-103		208	196	na	0	0	na						
FOV-001 (A11/A24)	Lck-208	na	164	85	249	0	0	158	14	10	na	PD	16.2	DOD
	Lck-486		0	243	1236	0	0	0						
	Lck-488		111	106	79 293	0	0	1729						
	PSMA-624		145	96	18 990	0	0	0						
	PTHrP-102		284	233	28 873	0	221	0						
FOV-002 (A24/A31)	EGF-R-800	na	0	178	78	0	0	0	13	14	9	SD	39.3	DOD
	MRP3-503		27	20	44 357	0	710	256						
	MRP3-1293		47	50	0	0	0	0						
	Lck-488		201	159	782	0	0	0						
	EZH2-735		0	91	0	0	0	0						
	PTHrP-102		55	85	25 414	0	424	0						
FOV-003 (A24)	Lck-208	na	77	75	23 700	0	0	0	9	9	14	PD	20	DOD
	PAP-213		0	68	9029	0	0	0						
	EGF-R-800		58	36	137	0	0	0						
	EZH2-735		34	26	22	223	0	0						
	PTHrP-102		47	40	12	0	0	779						
FOV-004 (A26/A33)	SART3-109	na	0	52	na	0	0	na	3	4	na	PD	5.3	DOD
	SART3-511		47	39	na	0	0	na						
	Lck-449		81	75	na	0	0	na						
	HNRPL-501		56	236	na	0	113	na						
FOV-005 (A2/A11)	HNRPL-501	na	15	0	1661	0	65	158	5	2	16	PD	11.4	DOD
	UBE2V-85		11	0	61	0	0	0						
	SART3-302		22	0	815	0	0	0						
	SART3-511		24	16	42	0	0	0						
	Lck-449		36	31	51 851	0	104	0						
FOV-006 (A11/A31)	SART3-109	na	0	22	138	0	0	0	2	3	6	SD	34.7	DOD
	SART3-511		13	14	569	0	0	0						
	SART3-734		0	0	180	0	0	0						
	Lck-449		41	37	72 533	107	107	199						
FOV-008 (A24)	SART2-93	na	63	35	0	0	0	54	8	4	0	PD	14.1	DOD
	Lck-208		69	13	0	0	0	0						
	EGF-R-800		70	58	0	0	0	74						
	SART2-161		65	0	0	0	0	0						
	PTHrP-102		60	24	0	0	0	0						
FOV-009 (A24/A26)	SART2-93	na	271	255	0	0	0	133	8	12	9	PD	8.1	DOD
	SART3-109		0	335	6242	0	0	0						
	Lck-208		264	236	13	0	0	0						
	EGF-R-800		455	428	0	0	0	0						
	SART2-161		441	475	882	0	0	0						
FOV-010 (A2/A24)	CypB-129	na	11	0	146	0	0	0	4	24	22	PD	39.9	AWD
	Lck-246		0	109	55 146	0	0	0						
	HNRPL-140		15	16	11 185	236	233	1080						
	SART3-109		0	45	923	0	0	0						
	PAP-213		14	14	4559	0	0	615						
	SART2-161		17	21	31	67	0	362						
	Lck-486		0	50	2961	0	0	0						
	Lck-488		0	76	67 229	0	0	0						
FOV-012 (A2/A11)	Lck-422	na	33	43	0	0	0	0	7	6	5	PD	16.1	DOD
	ppMAPkkk-432		44	0	29	0	0	57						
	HNRPL-501		0	112	128	0	0	0						
	UBE2V-43		147	0	0	0	0	0						
	SART3-109		3376	5187	44 517	0	0	0						
	SART3-511		169	146	156	0	0	0						
FOV-013 (A24)	SART3-109	na	561	602	na	0	65	na	6	6	na	PD	4	DOD
	Lck-208		96	0	na	0	0	na						
	MRP3-503		19	21	na	0	0	na						
	SART2-161		44	43	na	0	0	na						
	Lck-486		403	312	na	0	0	na						

(*continued*)

Table 4. Continued

ID (HLA-A type)	Peptides for vaccination	Antigen expression in tumor tissue	IgG response (FIU)			CTL response (IFN-γ producing cells/10^5 cells)			Numbers of IgG positive peptides			Best clinical response	Overall survival (months)	Prognosis
			Pre	6th	12th	Pre	6th	12th	pre	6th	12th			
FOV-014 (A24)	SART3-109	na	1692	1899	1298	0	0	0	4	5	4	PD	32.2	DOD
	PAP-213		11	0	23	0	0	806						
	SART2-161		17	20	0	0	0	200						
	Lck-486		1528	1550	1423	0	0	0						
	Lck-488		0	18	0	0	0	0						
	PTHrP-102		0	0	11	0	0	0						
FOV-015 (A2/A31)	CypB-129	na	16	16	0	0	0	0	7	7	3	SD	10.7	DOD
	Lck-422		49	0	0	0	0	0						
	ppMAPkkk-432		29	24	0	0	0	0						
	HNRPL-501		108	81	168	0	0	0						
	SART3-109		1811	1934	148773	na	na	na						
	SART3-511		100	106	16	na	na	na						
FOV-016 (A24/A31)	SART3-109	na	1419	635	0	0	0	68	6	3	4	PD	29.5	DOD
	Lck-486		353	537	271127	0	0	217						
	Lck-488		11	0	423	0	0	0						
	SART3-511		40	0	0	0	0	0						
	PAP-248		24	12	0	0	0	0						
	Lck-486		85	na	na	0	na	na						
FOV-019 (A24/A33)	SART3-109	3+	37	0	0	0	0	0	3	2	5	PD	32.8	AWD
	Lck-486	0	1474	1528	12953	0	0	0						
	PAP-248	0	27	0	15	0	0	0						
FOV-022 (A24/A33)	SART2-93	na	39	32	6554	0	341	274	12	9	11	PD	22	DOD
	PAP-213		69	0	17580	0	502	0						
	SART3-511		82	59	76	0	0	0						
	CypB-129		289	276	811	182	0	0						
	WHSC2-103		138	143	9025	0	0	0						
FOV-023 (A24)	PAP-213	na	25	464	na	0	361	na	2	3	na	PD	16.8	DOD
	Lck-486		312	5553	na	0	0	na						
FOV-024 (A2/A24)	HNRPL-501	na	823	241	na	0	0	na	7	3	na	PD	8.7	DOD
	PAP-213		11	0	na	0	0	na						
	PSA-248		14	23	na	0	0	na						
	Lck-486		18	20	na	0	0	na						
FOV-026 (A2/A11)	Lck-246	na	662	3179	23675	0	1119	1993	16	17	19	PD	17.6	AWD
	WHSC2-141		63	5223	52601	0	2863	1214						
	SART3-302		351	26824	27041	0	928	143						
	SART3-309		48	295	4421	0	2233	506						
FOV-027 (A2/A24)	ppMAPkkk-432	na	121	127	162	0	0	0	17	18	21	CR	15.2	AWD
	SART3-302		474	4297	15968	481	314	0						
	PAP-213		104	96	37274	0	0	109						
	EGF-R-800		34	34	882	0	0	0						
	Lck-488		42	47	502	0	0	202						
FOV-028 (A24)	SART2-93	3+	56	58	65	0	0	179	12	10	10	PD	14.2	AWD
	EGF-R-800	1+	126	120	3832	0	355	140						
	Lck-486	0	28	33	106	0	0	0						
	Lck-488	0	57	63	2425	0	68	541						
	PSMA-624	0	16	17	72	0	0	0						
	PTHrP-102	3+	50	54	58	0	0	900						
FOV-030 (A24/A31)	SART2-93	3+	49	65	356	0	0	86	16	19	20	PD	16.5	AWD
	PAP-213	1+	190	335	10874	0	0	216						
	PSA-248	0	41	7738	9876	0	0	0						
	Lck-488	0	68	97	9767	0	0	1555						
FOV-031 (A11/A26)	SART3-734	na	704	807	914	0	0	0	7	7	8	PD	15.5	AWD
	Lck-449		303	344	577	0	0	0						
	PAP-248		711	728	759	0	0	0						
	WHSC2-103		443	498	1771	0	0	0						
	ppMAPkkk-432		416	364	337	0	0	0						
FOV-032 (A11/A24)	SART2-93	na	36	34	225	0	0	315	15	13	19	PD	11.6	AWD
	Lck-208		39	0	28	0	0	0						
	Lck-488		27	29	139	0	0	0						
	PSMA-624		188	193	10643	0	0	0						
FOV-033 (A24)	SART2-93	na	483	642	11561	109	357	0	8	10	9	PD	10.7	AWD
	PAP-213		125	10222	16907	0	0	0						
	Lck-486		127	146	11828	0	0	0						
	Lck-488		239	1296	1374	0	0	0						
FOV-034 (A31/A33)	SART3-511	na	49	41	298	0	0	0	5	5	7	PD	10.5	AWD
	SART3-734		4398	26909	26716	0	0	0						
	PAP-248		112	355	322	0	0	0						

(*continued*)

ID (HLA-A type)	Peptides for vaccination	Antigen expression in tumor tissue	IgG response (FIU) Pre	6th	12th	CTL response (IFN-γ producing cells/10^5 cells) Pre	6th	12th	Numbers of IgG positive peptides pre	6th	12th	Best clinical response	Overall survival (months)	Prognosis
	WHSC2-103		74	192	582	0	0	0						
FOV-036 (A24/A31)	SART2-93	na	184	491	354	0	0	0	20	22	20	PD	13.8	AWD
	SART3-109		30	3314	204	0	0	0						
	PAP-213		99	19701	20055	114	377	244						
	Lck-486		240	713	1672	0	0	147						
	Lck-488		618	1571	20091	0	340	0						
	PSMA-624		26	21092	15900									
FOV-037 (A24/A31)	SART2-93	na	27	27	20	0	0	na	10	7	na	PD	4.6	DOD
	Lck-486		25	25	17	0	0	na						
	Lck-488		31	31	21	193	0	na						
	PTHrP-102		11	11	0	150	0	na						
FOV-038 (A2/A24)	SART2-93	na	139	85	na	0	0	na	19	18	na	PD	7.9	DOD
	SART2-161		0	39	na	0	0	na						
	Lck-486		217	113	na	0	0	na						
	Lck-488		338	189	na	0	0	na						
	PSMA-624		52	29	na	0	0	na						
FOV-039 (A24/A26)	SART2-93	na	130	125	15304	984	760	599	8	6	8	PD	10.3	AWD
	SART3-109		54	42	54	800	392	0						
	PAP-213		65	52	15235	840	241	0						
	Lck-488		119	69	8511	784	0	573						
FOV-040 (A11/A26)	SART3-511	na	6092	2011	na	0	0	na	8	8	na	PD	2.2	DOD
	SART3-734		3012	1991	na	0	0	na						
	Lck-90		52	46	na	0	0	na						
	PAP-248		72	33	na	0	0	na						
	WHSC2-103		104	59	na	0	0	na						
FOV-041 (A31/A33)	Lck-90	na	27	19	758	0	0	88	4	4	5	PD	12.5	AWD
	Lck-449		106	96	13170	189	0	164						
	CypB-129		18	18	24099	0	0	0						
	WHSC2-103		111	101	193	429	133	0						
FOV-042 (A31/A33)	SART3-511	na	141	65	64	0	0	46	7	7	7	PD	9.5	AWD
	Lck-90		542	407	327	0	0	0						
	CypB-129		115	98	16114	0	0	0						
	WHSC2-103		160	149	524	0	79	0						
FOV-043 (A11)	SART3-511	na	0	268	16065	0	0	0	4	6	4	PD	12.2	AWD
	SART3-734		375	1075	7041	0	191	0						
	Lck-449		134	364	3980	0	0	215						
	PAP-248		154	136	4479	0	0	0						
	WHSC2-103		141	89	0	0	0	0						
FOV-044 (A24/A31)	SART2-93	na	47	80	194	0	368	0	9	7	16	PD	12.2	AWD
	SART2-161		0	129	1728	0	0	0						
	Lck-486		17	3983	30429	0	0	0						
	Lck-488		30	399	52865	0	454	0						
	SART3-511		0	0	116	0	0	0						
	Lck-449		45	0	21890	0	221	0						
	WHSC2-103		40	0	1000	0	0	0						
FOV-045 (A11/A24)	SART2-93	na	15	11	na	0	0	na	7	8	na	PD	8.3	DOD
	SART3-109		17	14	na	0	513	na						
	Lck-488		29	25	na	0	45	na						
	SART3-511		61	61	na	0	0	na						
	SART3-734		71	51	na	0	0	na						
FOV-046 (A11/A24)	SART2-93	na	103	8781	na	59	851	na	4	4	na	PD	6.6	DOD
	Lck-488		17	143	na	0	649	na						
	Lck-449		21	15066	na	0	388	na						
	WHSC2-103		32	0	na	0	0	na						
FOV-047 (A24/A33)	SART2-93	na	167	154	192	0	0	85	16	14	21	PD	8.4	AWD
	MRP3-1293		37	33	52	0	0	0						
	Lck-486		49	34	13420	0	0	79						
	Lck-488		159	133	18116	0	0	0						
FOV-048 (A2/A24)	EGF-R-800	na	162	155	na	0	0	na	21	18	na	PD	4.7	AWD
	Lck-488		599	4943	na	0	0	na						
	PSMA-624		198	297	na	0	0	na						
	PTHrP-102		138	141	na	0	0	na						
FOV-049 (A24)	SART2-93	na	236	282	3995	0	0	0	7	10	10	PD	10.8	AWD
	Lck-486		32	37	322	0	0	0						
	Lck-488		69	81	408	0	0	0						
	PSMA-624		24	40	798	0	0	0						

CR, complete response; SD, stable disease; PD, progress disease; AWD, alive with disease; DOD, died of disease; and na, not avairable.

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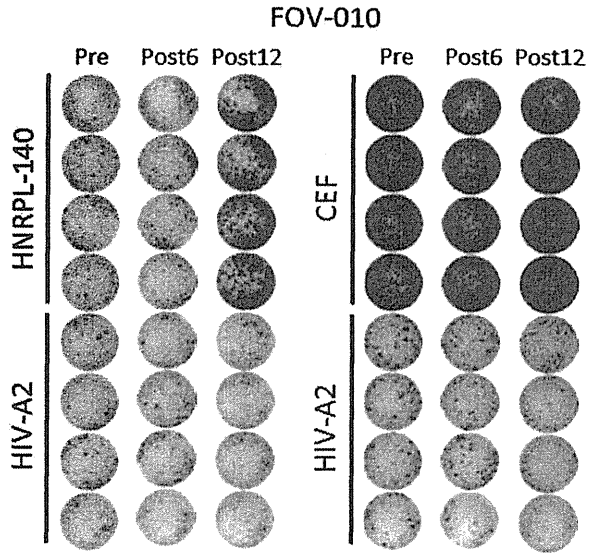

Figure 2. Representative well images of ELISPOT assay. HIV-A2 peptide was used as a negative control. CEF is a cocktail of CMV-, EBV- and Flu-peptides and used as a positive control.

plus chemotherapy. No patient had a partial response. Three patients (FOV-002, -006 and -015) had stable disease (SD). The remaining 21 patients had progressive disease (PD). The median survival time (MST) of the 42 recurrent patients was 19.1 months. Among them, the MST values of the 17 platinum-sensitive and 25 platinum-resistant recurrent cases were 39.3 and 16.2 months, respectively (Figure 4a). The MST values of PPV monotherapy or PPV in combination with any chemotherapy during the 1st to 12th vaccination for the total 42 cases were 20 and 19.1 months (Figure 4b), for the platinum-sensitive cases were 39.3 and 32.2 months (Figure 4c) and for the platinum-resistant cases were 16.8 and 16.1 months, respectively (Figure 4d).

Under these circumstances, the Cox proportional hazards model was used to determine whether immunological responses could be prognostic factors for OS (Table 5). The frequency of lymphocytes at the sixth vaccination and ES were significantly prognostic of OS ($p = 0.0029$ and $p = 0.0135$, respectively). Neither an increase in CTL nor an increase in IgG responses was significantly correlated to the OS, although augmentation of the IgG response was observed in each of the three SD and one CR cases and augmentation of the CTL response was observed in one CR and one SD case. For a better understanding of the involvement of these factors, a log-rank test was also used for the statistical analysis, and the patients with higher lymphocyte frequency or with ES at the sixth vaccination showed longer OS, respectively (Figure 5a and b). As a consequence, ED was inversely correlated with OS (ED + versus ED−, $p = 0.0797$; ED + versus ES, $p = 0.0247$) (Figure 5c and d). In contrast, age, PS and the number of previous regimens were not significantly prognostic of OS.

Discussion

The prognosis of recurrent ovarian cancer remains very poor, with an MST of 18–30 months in platinum-sensitive cases and 8–12 months in platinum-resistant cases[25–28]. Therefore, new innovative therapies are needed for the treatment of recurrent ovarian cancer. We conducted a phase II study of PPV for recurrent ovarian cancer from the viewpoint of OS. This study showed that the MST values of 17 cases of platinum-sensitive and 25 cases of platinum-resistant recurrent ovarian cancer treated with PPV were 39.3 and 16.2 months, respectively. These MST values were longer than the historical control values for recurrent ovarian cancer patients treated in our institution (the historical MST values for platinum-sensitive and -resistant cases were 23 and 8 months, respectively). These results suggest that PPV has the potential to prolong the OS of both platinum-sensitive and platinum-resistant cases.

Thirty-one of 37 cases in this study showed PD at the 12th vaccination, suggesting that PPV did not shrink the tumors but rather delayed the tumor progression, in agreement with the previously conducted PPV for patients with advanced cancers other than ovarian cancer[17–20].

Our previously conducted trials of PPV in various types of cancers also confirmed its safety[21]. PPV toxicity consisted mainly of skin reactions at the injection sites. Although PPV is considered to be feasible, one severe adverse event associated with vaccination was observed. This patient underwent pelvic lymphadenectomy for primary debulking surgery. Lower-limb lymphedema appeared along with a skin reaction at the vaccination sites. Thereafter, infection occurred. We concluded that the infection was associated with PPV. It would thus be better to avoid vaccination of the lower limbs in patients who have undergone pelvic lymphadenectomy. We also investigated the therapeutic potential of the combination of PPV plus chemotherapy. The MST values of PPV monotherapy or PPV in combination with any chemotherapy during the 1st to 12th vaccination of the platinum-sensitive cases were 39.3 and 32.2 months, and those of platinum-resistant cases were 16.8 and 16.1 months, respectively. The patients who could not tolerate concomitant chemotherapy received PPV monotherapy, and most of these patients underwent chemotherapy after completion of PPV. The boosting of immune responses began to be apparent at the 12th vaccination in the vast majority of patients in this study. Therefore, PPV monotherapy for the 1st to 12th vaccination followed by chemotherapy in platinum-sensitive cases could be recommended not only from a clinical but also an immunological point of view. In the platinum-resistant cases, we did not observed such a clear difference between the PPV monotherapy and the PPV in combination with chemotherapy. Therefore, in platinum-resistant cases, the combination of PPV plus chemotherapy might not contribute to better prognosis, and might increase adverse events. These issues, however, should be addressed in the next step of a clinical trial with relatively large numbers of patients.

Since only some of the patients showed clinical benefit from the peptide-based cancer vaccine, the identification of biomarkers to predict the OS is an important issue[29–32]. Increased IgG responses were observed in 38.1% and 96.7% of patients at the 6th and 12th vaccinations, suggesting that 12 vaccinations would be required to obtain the peptide-specific immunity by PPV. On the other hand, increased CTL responses were observed in 42.9% and 63.3% of patients at

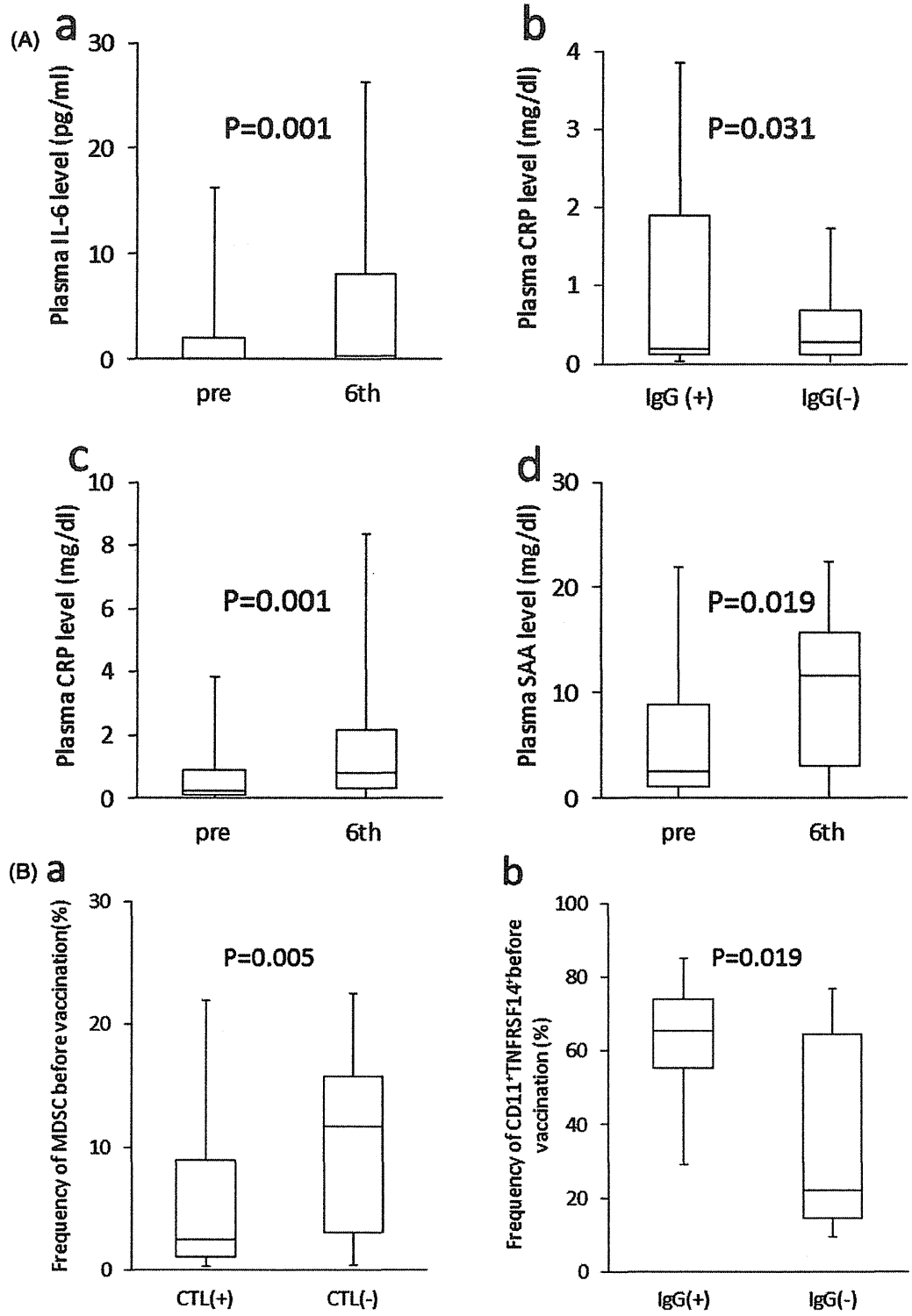

Figure 3. (A) Plasma levels of inflammatory cytokines. There were significant increases in IL-6, CRP, and SAA levels at the time of the 6th vaccination ($p=0.001$, $p=0.001$ and $p=0.01$). Plasma CRP levels before vaccination were higher in the group that showed an augmentation of peptide-specific IgG response at the sixth vaccination ($p=0.031$). (B) Flow-cytometric analysis of PBMCs. The frequency of MDSC in prevaccination PBMCs was lower in the group that showed an augmentation of CTL response at the sixth vaccination ($p=0.005$) (a). The frequency of CD11+TNFRSF14+ before vaccination was higher in the group that showed an augmentation of IgG response at the sixth vaccination ($p=0.019$) (b).

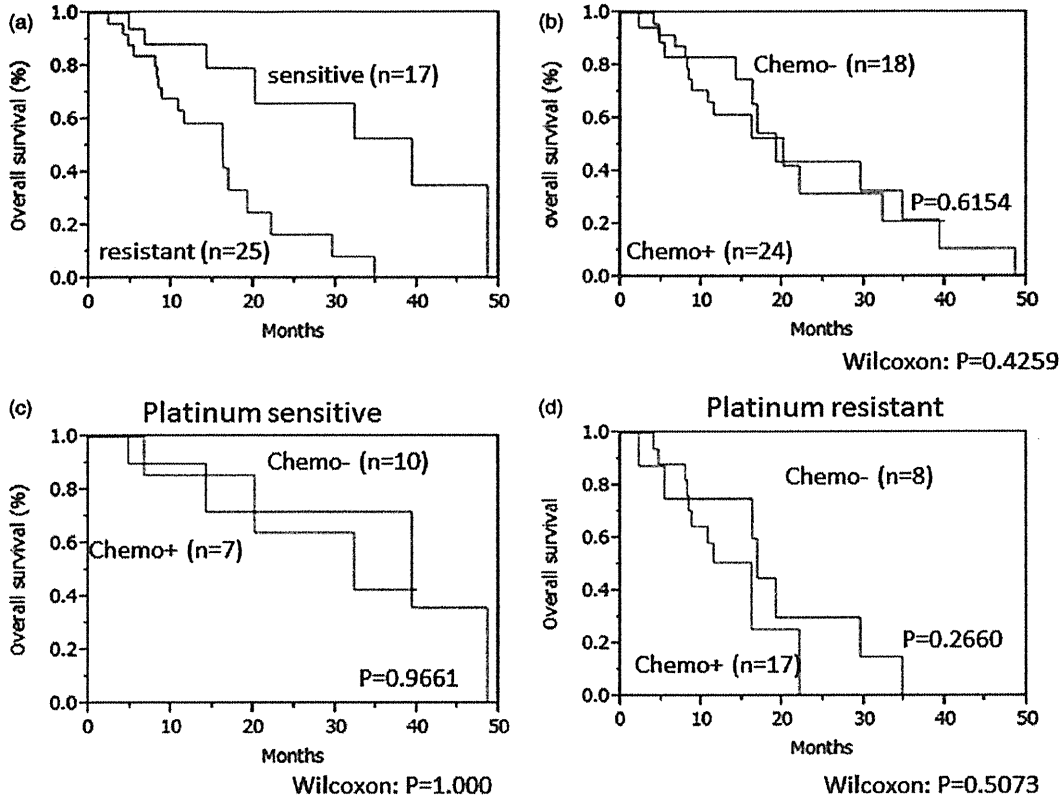

Figure 4. Kaplan–Meier analysis of overall survival. (a) The median survival times of platinum-sensitive (blue) and -resistant (red) recurrent cases were 1179 and 483 days, respectively. (b) The overall survival times of patients who underwent PPV with (red) and without (blue) chemotherapy were not significantly different ($p = 0.0941$, Log-rank test). (c) The overall survival times of platinum-resistant recurrent patients who underwent PPV with (red) and without (blue) chemotherapy were not significantly different ($p = 0.3497$, Log-rank test). (d) The overall survival times of platinum-sensitive recurrent patients who underwent PPV with (red) and without (blue) chemotherapy were not significantly different ($p = 0.2032$, Log-rank test).

Table 5. Univariate and multivariate analyses with clinical and immunological data and OS ($n = 42$).

	Univariate analysis	
Factors	Hazard ratio (95% CI)	p Value
Lymphocyte frequency (%) at pre-vaccination	0.967 (0.927–1.007)	0.1083
Lymphocyte frequency (%) at sixth vaccination	0.927 (0.874–0.976)	0.0029
Skin reaction at the injection	0.458 (0.163–1.474)	0.1771
Increase in CTL responses	0.659 (0.261–1.553)	0.3450
Increase in IgG responses	0.868 (0.241–2.509)	0.8045
epitope spreading	0.299 (0.095–0.789)	0.0135

CI, confidence interval and CTL, cytotoxic T lymphocytes.

the 6th and 12th vaccinations, respectively. The CTL response was less augmented at the 12th vaccination than the IgG response. This might reflect immunological anergy or suppression through MDSC or T-cell checkpoint molecules such as PD-1 or CTLA-4[11,33]. Indeed, we showed that MDSC could be involved in suppression of CTL induction. Repeated vaccination by the same epitope peptides may also induce T-cell exhaustion. We are currently conducting a clinical study in which different peptide sets will be used for each cycle of vaccination to determine whether the T cell exhaustion can be prevented by such a regimen.

Interestingly, ES was correlated with IgG and CTL responses at the 6th vaccination. Furthermore, ES was a prognostic factor by univariate analysis. These results indicated that PPV induced not only peptide-specific immunological boosting in response to the vaccinated peptides but also promoted the spreading of immune responses to the other TAA-derived peptides, which together resulted in the prolongation of OS. In contrast, ED was negatively correlated with OS. These results suggest that T-cell responses to large numbers of TAAs could be better than those to small numbers of TAAs. Further studies with large numbers of patients will be needed to confirm this point. It should be noted that IgGs to the CTL-epitope peptides may not reflect IgGs to the parental protein in most cases, since the peptide-specific IgG recognized a linear epitope but not a conformational epitope, and most of the linear epitopes are conformationally hidden within the molecules.

Although multivariate analysis was not performed due to the limited number of cases, univariate analysis revealed that the frequency of lymphocytes at the 6th vaccination and ES were correlated with unfavorable and favorable OS, respectively. More data still need to be collected to validate these findings. Evaluation of the identified factors could be useful for predicting whether individual patients with recurrent ovarian cancer would benefit from cancer vaccines. These factors might not necessarily be unique to the vaccinated patients.

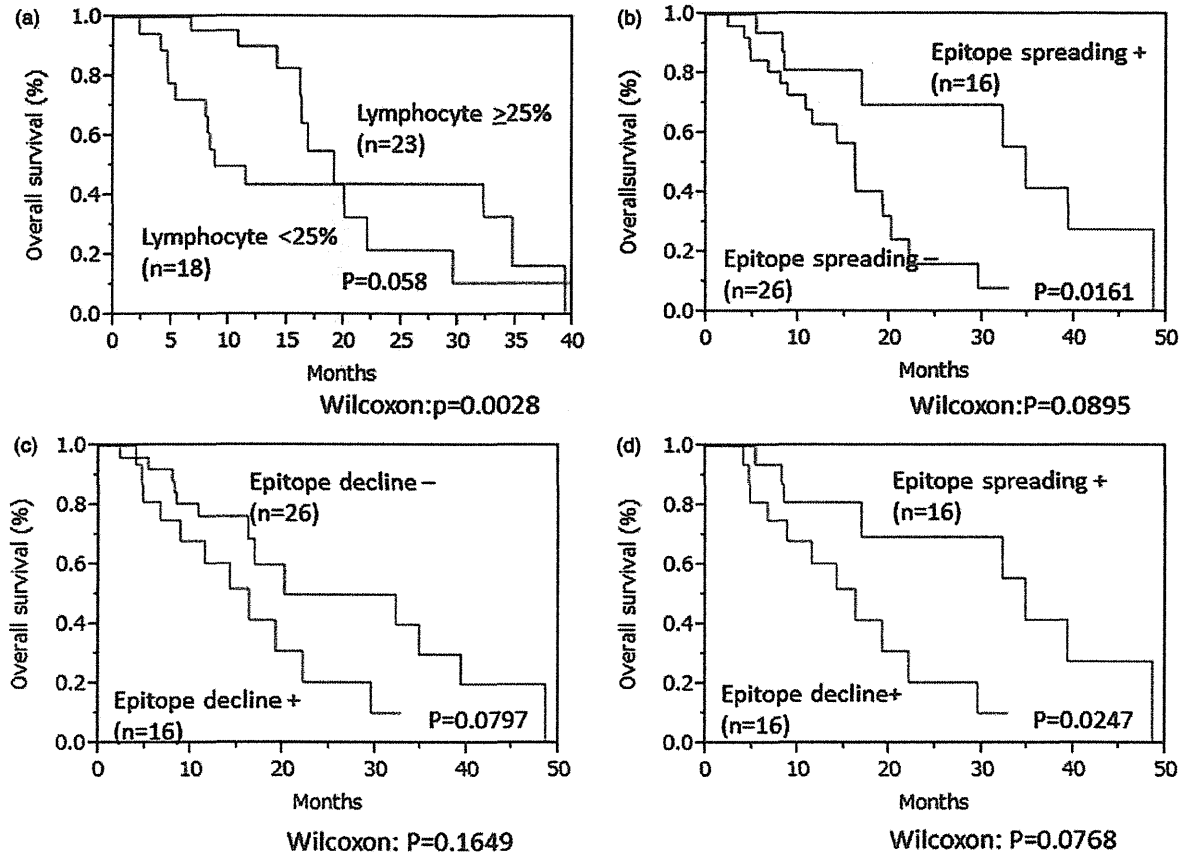

Figure 5. Post-vaccination biomarker analysis. The patients with a lymphocyte frequency >25% tended to have longer overall survival, although this association was not statistically significant ($p = 0.058$) (a). The patients who demonstrated epitope spreading at the sixth vaccination showed longer overall survival ($p = 0.0164$, Log-rank test) (b). The patients with epitope decline at the sixth vaccination showed shorter overall survival as compared to those without epitope decline ($p = 0.0798$, Log-rank test) (c) or those with epitope spreading ($p = 0.0247$, Log-rank test) (d).

Based on these findings on the safety, immune responses and possible prolongation of OS, the next stage of a clinical trial of PPV without chemotherapy during the 1st to 12th vaccination could be recommended for recurrent ovarian cancer patients.

Conclusion

A phase II study of PPV for recurrent ovarian cancer patients was performed to evaluate the efficacy from the point of view of OS. Boosting of CTL or IgG responses specific for the peptides used for vaccination was observed in the majority of patients without any vaccine-related systemic severe adverse events. The MST of the PPV monotherapy group was significantly longer than that of the PPV with chemotherapy group. Because of the safety and possible prolongation of OS, a clinical trial of PPV without chemotherapy during the 1st to 12th vaccination in recurrent ovarian cancer patients is merited.

Declaration of interest

Yamada is a part-time executive of the Green Peptide Co. All other authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Research Article

Cancer Immunology Research

Phase II Study of Personalized Peptide Vaccination for Previously Treated Advanced Colorectal Cancer

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Abstract

The prognosis of advanced colorectal cancer (aCRC) remains poor, and development of new therapeutic approaches, including immunotherapy, is needed urgently. Herein we report on our phase II study of personalized peptide vaccination (PPV) in 60 previously treated patients with aCRC, who had failed at least one regimen of standard chemotherapy and/or targeted therapy. For PPV, a maximum of four HLA-matched peptides were individually selected from a pool of 31 different peptide candidates based on preexisting host immunity, and administered subcutaneously without severe adverse events. Boosting of IgG and cytotoxic T lymphocyte (CTL) responses specific to the administered peptides was observed in 49% and 63%, respectively, of the patients, who completed the first cycles of six vaccinations. Median overall survival (OS) time was 498 days, with 1- and 2-year survival rates of 53% and 22%, respectively. Multivariate Cox regression analysis of prevaccination factors showed that plasma IL6, IP-10, and BAFF levels were significantly prognostic for OS [hazard ratio (HR), 1.508, $P = 0.043$; HR, 1.579, $P = 0.024$; HR, 0.509, $P = 0.002$, respectively]. In addition, increased peptide-specific CTL responses after vaccination were significantly predictive of favorable OS (HR, 0.231; $P = 0.021$), suggesting a causal relationship between biologic and clinical efficacy of PPV. On the basis of the safety profile and potential clinical efficacy, we believe that clinical trials of PPV would be warranted for previously treated patients with aCRC. *Cancer Immunol Res; 2(12); 1154–62. ©2014 AACR.*

Introduction

Colorectal cancer is one of the major causes of cancer-related death in the world. Although recent advances in chemotherapy and/or targeted therapy have helped to improve the clinical outcomes of patients with advanced colorectal cancer (aCRC), the prognosis still remains poor (1). Therefore, development of new therapeutic approaches, including immunotherapy, would be highly desirable. However, limited numbers of clinical trials of immunotherapies have been reported for patients with aCRC (2, 3).

We have developed a novel approach of cancer immunotherapy, named personalized peptide vaccination (PPV), in which vaccine peptides were selected from 31 cytotoxic T lymphocyte (CTL) epitope peptides derived from 15 tumor-associated antigens (TAA), based on both HLA class I types and preexisting host immunity (4, 5). Recently conducted clinical trials of PPV for patients with various types of cancers demonstrated the feasibility of this new approach (4–7). For patients with aCRC, phase I studies showed the safety and immunogenicity of PPV combined with chemotherapeutic agents, along with possible prolongation of survival time in immunologic responders (8, 9). We conducted a phase II study to examine the feasibility of PPV and to identify biomarkers that would be useful for prediction of overall survival (OS) in previously treated patients with aCRC.

Materials and Methods

Patients

Previously treated patients with aCRC, who had failed at least one regimen of standard chemotherapy and/or targeted therapy, were eligible for inclusion in this study, if they had positive humoral responses as determined by the peptide-specific IgG titers to at least two of the 31 different candidate vaccine peptides (Supplementary Table S1; refs. 4–9). Other inclusion and exclusion criteria are shown in Supplementary Methods. The protocol was approved by the Kurume University Ethics Committee and was registered in the University

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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Hospital Medical Information Network (UMIN) Clinical Trials Registry (UMIN000006493). After a full explanation of the protocol, written informed consent was obtained from all patients before enrollment.

Clinical protocol

This was an open-label phase II study in which the endpoints were to analyze the clinical feasibility and safety of PPV and to identify biomarkers useful for prediction of OS after PPV in patients with aCRC. Thirty-one vaccine peptide candidates, whose safety and immunologic effects had been confirmed in clinical studies conducted previously (4–9), were prepared under the conditions of Good Manufacturing Practice (GMP) by the PolyPeptide Laboratories and American Peptide Company. Expressions of vaccine antigens in colorectal cancer tissues were examined by immunohistochemistry (Supplementary Fig. S1). Of the 15 vaccine antigens used for PPV, 13 were detectable in colorectal cancer tissues tested, but not the two prostate-related antigens (PSA and PSMA; Supplementary Table S1).

The protocol consisted of two cycles of six vaccinations. Two to four HLA-matched peptides were selected from the 31 peptides in individual patients, based on preexisting host immunity before vaccination by assessing the titers of IgG specific to each peptide, as described previously (4–9). The peptides derived from PSA and PSMA were selected only when preexisting IgG responses to other remaining peptides were absent. The selected peptides (3 mg/each peptide) were administered subcutaneously with incomplete Freund adjuvant (Montanide ISA51; Seppic) once a week for 6 consecutive weeks. After the completion of the first cycle of six vaccinations, IgG titers specific to each of 31 peptide candidates in plasma from vaccinated patients were measured again, and two to four HLA-matched peptides with higher specific IgG titers were selected and administered six times every 2 weeks for the second vaccination cycle. After the second cycle, vaccinations were maintained, if the patients wished; two to four antigen peptides, which were reselected on the basis of the titers of peptide-specific IgG at every cycle of six vaccinations, were administered every 4 weeks until uncontrollable disease progression. Combined chemotherapies and/or targeted therapies were allowed during the vaccination period. Adverse events (AE) were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 4.0. Complete blood counts and serum biochemistry tests were performed before and after every six vaccinations.

Measurement of humoral and cellular immune responses

Peripheral blood (30 mL) was obtained from the vaccinated patients before and after each cycle of six vaccinations. After centrifugation, plasma was separated and stored frozen until analysis. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare) and stored frozen until analysis. Postvaccination blood samples were available from 51 and 35 patients at the end of the first (6 vaccinations) and second (12 vaccinations) cycles, respectively.

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), as reported previously (10, 11). CTL responses specific to the vaccine peptides were evaluated by the IFNγ ELISPOT assay. The detailed procedures are shown in the Supplementary Methods. When spot numbers in response to specific peptides were significantly higher ($P < 0.05$ by Student t test) than those in response to the control peptides, antigen-specific CTL responses were shown as the differences between them (means of the triplicate samples).

Measurement of laboratory markers

Levels of C-reactive protein (CRP), serum-amyloid A (SAA), and IL6 in prevaccination plasma were examined by ELISA using kits from R&D Systems, Life Technologies, and eBioscience, respectively. Bead-based multiplex assays were used to measure cytokines, including IL4, IL13, IL21, IP-10 (IFNγ-induced protein 10), BAFF (B-cell activating factor), and TGFβ, with the Luminex 200 system. Prevaccination plasma from 1 patient was unavailable for this analysis ($n = 59$). Frozen plasma samples were thawed, diluted, and assayed in duplicate in accordance with the manufacturer's instructions. Means of the duplicate samples were used for statistical analysis.

IL6, IL6 receptor (IL6R), and CRP genetic polymorphisms

DNA was extracted from thawed PBMCs using a QIAamp Blood kit (Qiagen) and stored at −80°C until analysis. To investigate the IL6 −634G>C (rs1800796), CRP 1846C>T (rs1205), and IL6R 48892A>C (rs8192284, Asp358Ala) genetic polymorphisms with the extracted DNA, genotyping was performed using the polymerase chain reaction-restriction fragment length polymorphism method, as reported previously (12, 13).

Statistical analysis

OS time was defined as duration from the first date of peptide vaccination or that of the first-line chemotherapy until the date of death and was censored by the last date of contact for patients alive at the last follow-up. The survival function, including survival rates, for OS was estimated by the Kaplan–Meier method with the Greenwood variance estimates. In addition, exploratory analyses, which were not predefined in the protocol, were performed to examine association among biomarkers, immune responses, and OS. Association between prevaccination biomarkers and OS were evaluated by univariate and multivariate analyses with the Cox proportional hazards regression model. In applying Cox regression, the transformation of log(biomarker + 1) was used because the distribution of each biomarker was highly skewed. Statistically significant biomarkers ($P < 0.1$) in the univariate analysis were included in the multivariate analysis. The Spearman rank correlation among these biomarkers was estimated to avoid collinearity.

Humoral and cellular immune responses were determined by IgG and CTL responses specific to the administered peptides, respectively. IgG responses were defined as positive if IgG titers specific to at least one of the administered peptides in the postvaccination plasma were more than two times higher than those in the prevaccination plasma, and as negative otherwise. CTL responses were defined as positive if CTL responses to at least one of the administered peptides in the postvaccination PBMCs were greater than those in the prevaccination PBMCs and as negative otherwise. Association between IgG or CTL responses and other prognostic factors was examined by logistic regression analysis. Association between IgG or CTL responses and OS was examined by the Kaplan–Meier method with the log-rank test and the Cox regression analysis. The relationship between IgG and CTL responses was evaluated by the χ^2 test. The prognostic significance of genetic polymorphisms was analyzed by the Kaplan–Meier survival curves with the log-rank test. All statistical tests were conducted at the two-sided 5% significance level, unless indicated. Because of the exploratory nature of biomarker analyses, any multiplicity adjustment was not applied. All statistical analyses were conducted using the JMP version 10 or SAS version 9.3 software package (SAS Institute Inc.).

Results

Patient characteristics

Between January 2009 and November 2012, 60 patients with aCRC were enrolled in this study. Table 1 summarizes the clinicopathologic characteristics of the enrolled patients. There were 33 male and 27 female subjects with a median age of 60 years, ranging from 35 to 83 years. All patients (stage IV, $n = 26$; recurrent, $n = 34$) were refractory to at least one regimen of chemotherapies and/or targeted therapies. The location of original tumor was right-sided colon ($n = 14$) or left-sided colon/rectum ($n = 46$). All patients had metastatic tumors; liver ($n = 33$), lung ($n = 31$), peritoneal dissemination ($n = 23$), or lymph nodes ($n = 14$). The number of metastatic organs per patient was one ($n = 29$), two ($n = 21$), or three ($n = 10$). Before enrollment, the patients had failed to respond to one ($n = 17$), two ($n = 15$), three ($n = 9$), four ($n = 13$), or five ($n = 6$) regimens of chemotherapies, targeted therapies, and/or combinations of them. The median duration of these preceding regimens before PPV was 552.5 days, ranging from 9 to 1,819 days. The median time from patient enrolment to first vaccination was 13.5 days, ranging from 7 to 27 days. The numbers of peptides used for vaccination during the first cycle were four peptides in 36 patients, three in 16 patients, and two in 8 patients. Among the 60 patients, 51 (85%) completed the first cycle of six vaccinations, and the remaining 9 patients failed to do so due to rapid disease progression. The median number of vaccinations was 12, with a range of 2 to 33. During the PPV, 49 patients (82%) received combined chemotherapies and/or targeted therapies, including FOLFOX/XELOX with bevacizumab ($n = 10$), FOLFIRI with bevacizumab ($n = 5$), FOLFIRI ($n = 5$), S-1 ($n = 5$), irinotecan with cetuximab ($n = 5$), cetuximab ($n = 5$), FOLFOX/XELOX ($n = 2$), FOLFIRI with cetuximab ($n = 2$), or other regimens ($n = 10$). The remaining 11 patients (18%) had no options for combined chemotherapies or were unable to tolerate them.

Table 1. Patient characteristics

Factor	Number
Age, years	
Median (range)	60 (35–83)
Gender	
Male	33
Female	27
Stage	
Stage IV	26
Recurrent	34
Location of original tumors	
Right-sided colon	14
Left-sided colon or rectum	46
Location of metastatic tumors	
Liver	33
Lung	31
Peritoneal dissemination	23
Lymph nodes	14
Number of metastatic organs	
1	29
2	21
3	10
Number of previous regimens	
1	17
2	15
3	9
4	13
5	6
Duration of previous treatments, days	
Median (range)	552.5 (9–1,819)
HLA type	
A2	19
A3	3
A11	16
A24	41
A26	10
A31	4
A33	11
Time from patient enrolment until first vaccination	
Median (range)	13.5 (7–27)
Number of vaccinations	
Median (range)	12 (2–33)
OS time, days	
Median (95% CI)	498 (223–654)

Adverse events

Toxicities are shown in Supplementary Table S2. The most frequent AEs were dermatologic reactions at the injection sites ($n = 55$; 92%), anemia ($n = 27$; 45%), lymphopenia ($n = 23$; 38%), and hypoalbuminemia ($n = 20$; 33%). Grade 4 anemia was noted in 2 patients. Grade 3 serious AEs (SAE) comprised

leukocytopenia ($n = 3$), lymphopenia ($n = 2$), increased γ-glutamyl transpeptidase (GGT; $n = 2$), hyponatremia ($n = 2$), ileus ($n = 2$), increased aspartate aminotransferase (AST; $n = 1$), hyperglycemia ($n = 1$), hypercholesteremia ($n = 1$), and rash ($n = 1$). However, according to the evaluation by the independent safety evaluation committee for this trial, all the grade 3 or 4 SAEs were concluded to be not directly associated with the vaccinations, but with other causes, such as combined chemotherapies and/or targeted therapies and cancer progression.

Clinical outcomes

Median OS time (MST) for the 60 patients from the first vaccination was 498 days [95% confidence interval (CI), 233–654 days] with 1- and 2-year survival rates of 53% and 22%, respectively (Fig. 1A). When calculated from the first date of the first-line chemotherapy, MST was 1,179 days (95% CI, 885–1,272 days) with 1-, 2-, 3-, 4-, and 5-year survival rates of 97%, 77%, 53%, 24%, and 15%, respectively (data not shown). Of note, among the enrolled 60 patients, 32 patients, who had a treatment history of two or more regimens of standard chemotherapy and were refractory or intolerant to all of irinotecan, oxaliplatin, and fluoropyrimidines before enrollment, showed MST of 375 days (95% CI, 191–561 days) from the first vaccination, with 1-year survival rate of 51% (Fig. 1B).

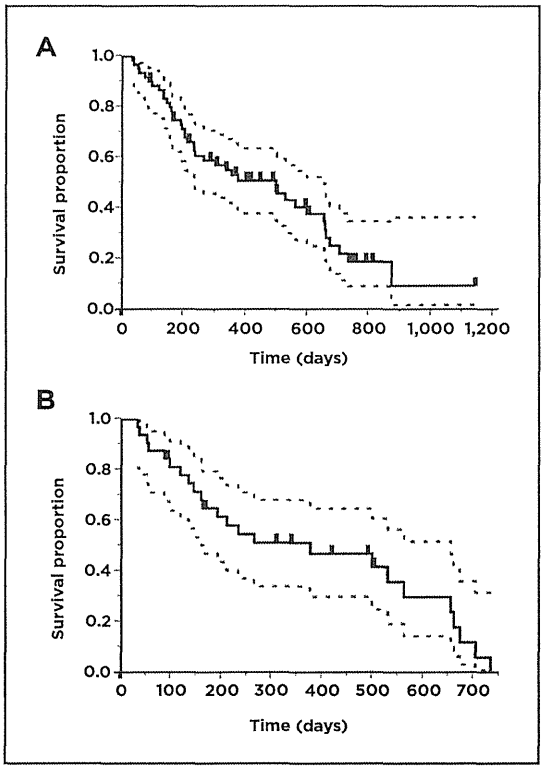

Figure 1. Kaplan–Meier survival analysis. Curves for OS (solid line) after PPV treatment were estimated by the Kaplan–Meier method in all 60 enrolled patients (A) and in 32 heavily treated patients who were refractory or intolerant to all of irinotecan, oxaliplatin, and fluoropyrimidines before enrollment (B). Dotted lines show 95% CIs. Censored patients are shown as vertical bars.

Relationship between prevaccination clinical findings or laboratory data and OS

The Cox proportional hazards model was used to identify factors that were significantly associated with OS, from prevaccination clinical findings or laboratory data. As shown in Table 2, univariate analysis using prevaccination clinical findings showed that the number of previous chemotherapy regimens were potentially prognostic factors ($P = 0.067$). In addition, albumin, carcinoembryonic antigen (CEA), CRP, SAA, IL6, IP-10, and BAFF in prevaccination blood were significantly prognostic of OS by univariate analysis ($P = 0.012$, $P = 0.002$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P = 0.018$, and $P = 0.005$, respectively). However, none of the other factors examined were significantly correlated with OS.

Multivariate Cox regression analysis was performed to evaluate the influence of each of the factors that had been shown to be significantly associated with OS in the univariate analysis ($P < 0.1$). SAA and CRP were not included in this analysis, because the level of SAA and CRP was highly correlated with that of IL6 (SAA vs. IL6: Spearman rank correlation coefficient = 0.482; CRP vs. IL6: Spearman rank correlation coefficient = 0.653). As shown in Table 2, higher IL6 and IP-10 levels and a lower BAFF level in prevaccination plasma were significantly predictive of unfavorable OS [hazard ratio (HR) for the unit of 1 SD, 1.508, 95% CI, 1.014–2.245, $P = 0.043$; HR, 1.579, 95% CI, 1.062–2.347, $P = 0.024$; HR, 0.509, 95% CI, 0.329–0.787, $P = 0.002$, respectively]. The other factors showed no statistically significant association.

Relationship between IL6, IL6R, or CRP genetic polymorphisms and OS

Because inflammation markers, IL6 and CRP, were potentially prognostic in patients treated with PPV, we examined genetic polymorphisms of related genes, IL6 −634G>C, CRP 1846C>T, and IL6R 48892A>C (Supplementary Table S3). There was no statistically significant relationship between IL6 634G>C polymorphism and OS ($P = 0.319$). However, CRP 1846C>T and IL6R 48892A>C polymorphisms tended to show a statistically significant effect on OS ($P = 0.069$ and 0.085, respectively). Patients carrying the CRP 1846C/C genotype had a potentially better prognosis than those carrying the CRP 1846C/T or those carrying the CRP 1846T/T genotype ($P = 0.029$ or 0.054, respectively; Fig. 2A). In addition, patients carrying the IL6R 48892C/C or 48892A/C genotypes tended to show a better prognosis than those carrying the IL6R 48892A/A genotype ($P = 0.059$; Fig. 2B). This genetic polymorphism was further evaluated in patients positive or negative for IL6 in prevaccination plasma (Fig. 2C). Of note, the difference between patients carrying the IL6R 48892C/C or A/C genotypes and the IL6R 48892A/A genotype was statistically significant in patients negative for plasma IL6 ($P = 0.025$), but not in those positive for plasma IL6 ($P = 0.118$).

Table 2. Univariate and multivariate analysis for OS with prevaccination clinical findings or laboratory data

	Univariate analysis		Multivariate analysis	
Factor	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age	1.000 (0.972–1.029)	0.991		
Gender (male vs. female)	1.626 (0.856–3.090)	0.138		
Stage (stage IV vs. recurrent)	1.173 (0.622–2.212)	0.623		
Number of previous chemotherapy regimens	1.249 (0.985–1.584)	0.067	1.279 (0.927–1.764)	0.134
Lymphocyte frequency, %	0.855 (0.661–1.172)	0.238		
Hemoglobin, g/dL	0.834 (0.628–1.108)	0.211		
Albumin, g/dL	0.677 (0.501–0.916)	0.012	0.805 (0.451–1.437)	0.462
Creatinine, mg/dL	1.075 (0.779–1.485)	0.659		
CEA, ng/dL	1.754 (1.240–2.483)	0.002	1.429 (0.938–2.177)	0.096
CRP, ng/mL	2.525 (1.590–4.011)	<0.001		
SAA, ng/mL	2.089 (1.433–3.046)	<0.001		
IL4, pg/mL	0.928 (0.667–1.292)	0.660		
IL6, pg/mL	1.890 (1.380–2.588)	<0.001	1.508 (1.014–2.245)	0.043
IL13, pg/mL	0.963 (0.660–1.405)	0.846		
IL21, pg/mL	1.206 (0.909–1.600)	0.193		
IP-10, pg/mL	1.518 (1.075–2.142)	0.018	1.579 (1.062–2.347)	0.024
BAFF, pg/mL	0.599 (0.421–0.853)	0.005	0.509 (0.329–0.787)	0.002
TGFβ, pg/mL	1.222 (0.861–1.736)	0.261		

Immune responses to the vaccine peptides

IgG responses specific to at least one of the administered peptides were increased in 25 of 51 patients (49%) and in 33 of 35 patients (94%) at the end of the first and second cycles of vaccinations, respectively (Supplementary Table S4). CTL responses specific to at least one of the administered peptides that were evaluated by IFNγ ELISPOT assay were increased in 32 of 51 patients (63%) at the end of the first cycle of vaccinations (Supplementary Table S4). A representative result of IFNγ ELISPOT assay with PBMCs before and after vaccination is shown in Fig. 3A. According to the χ^2 test, increased CTL responses against administered peptides after the first cycle of vaccinations were significantly associated with increased IgG responses ($P = 0.002$).

Relationship between the increase in peptide-specific CTL or IgG responses after vaccination and other potential prognostic factors, including prevaccination IL6, IP-10, and BAFF levels (Table 2), were examined by logistic regression analysis. As shown in Table 3, the level of IP-10 was predictive of the increase in CTL and IgG responses (OR, 0.427; 95% CI, 0.191–0.957; $P = 0.039$; OR, 0.354; 95% CI, 0.127–0.982; $P = 0.046$; respectively), whereas other factors, including IL6 and BAFF levels, were not predictive.

Prognostic significance of boosting of peptide-specific CTL and IgG responses

The prognostic significance of successful boosting of peptide-specific CTL or IgG responses was analyzed by the Kaplan–Meier survival curves with the log-rank test. This analysis showed a statistically significant association between increased CTL or IgG responses and OS ($P = 0.025$ and 0.022, respectively; Fig. 3B and C). Patients with both CTL and IgG responses ($P = 0.010$), but not those with CTL responses alone ($P = 0.138$) or IgG responses alone ($P = 0.351$), showed significantly better prognosis than those without CTL or IgG responses (Supplementary Fig. S2).

In addition, multivariate Cox regression analysis with peptide-specific CTL or IgG responses (positive or negative) and other potential prognostic factors (Table 2) was performed. IP-10 was not included in this analysis because the CTL and IgG responses were significantly associated with plasma IP-10 level (Table 3). As shown in Table 4, increased CTL responses after vaccination were significantly associated with favorable OS (HR, 0.231; 95% CI, 0.067–0.803; $P = 0.021$) independently of other factors, whereas IgG responses after vaccination were not significantly predictive of favorable OS (HR, 0.790; 95% CI, 0.285–2.188; $P = 0.650$). Furthermore, to analyze association of the magnitude of CTL responses with OS, the number of peptides, to which CTL responses were increased after vaccination, was evaluated by multivariate analysis. As shown in Supplementary Table S5, the number of peptides with increased CTL responses after vaccination was also significantly predictive of favorable OS (HR, 0.216; 95% CI, 0.077–0.604; $P = 0.004$).

Discussion

In this study, we demonstrated that successful boosting of peptide-specific CTL responses resulted in increased OS after PPV, suggesting a potential clinical benefit of PPV. The most unique aspect of PPV is the personalized selection of optimal antigen peptides for individual patients on the basis of preexisting host immunity before vaccination (4, 5). In view of the heterogeneity of tumors and the complexity and diversity of

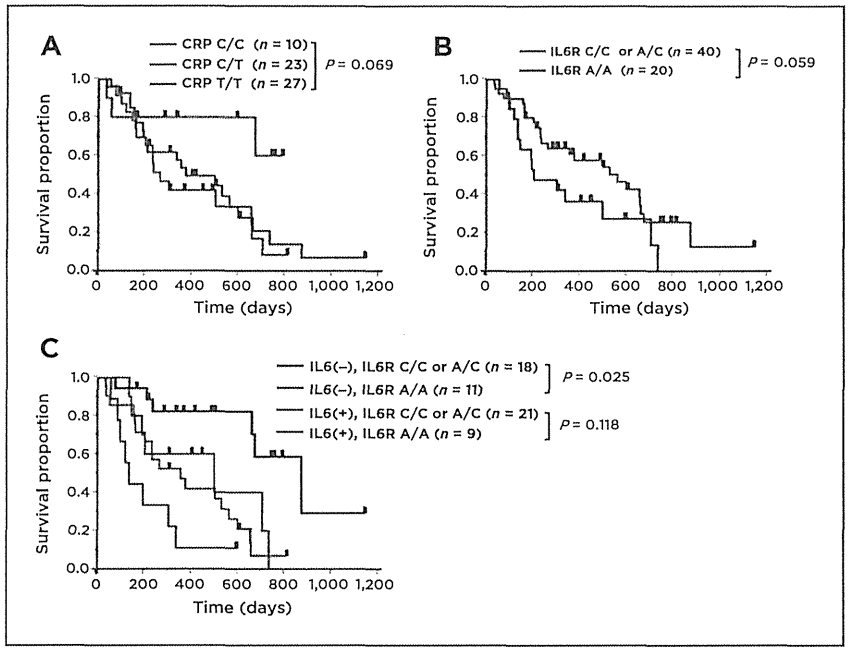

Figure 2. Prognostic significance of CRP 1846C>T and IL6R 48892A>C polymorphisms in patients with aCRC treated with PPV. To examine the prognostic significance of CRP 1846C>T and IL6R 48892A>C polymorphisms in patients with aCRC treated with PPV, curves for OS were estimated by the Kaplan–Meier method, and differences between survival curves were statistically analyzed using the log-rank test. Censored patients are shown as vertical bars. A, patients treated with PPV were divided into three subgroups according to CRP 1846C>T polymorphisms [CRP 1846C/C ($n = 10$), C/T ($n = 23$), T/T ($n = 27$)]. B, patients treated with PPV were divided into two subgroups according to the IL6R 48892A>C polymorphisms [IL6R 48892C/C or A/C ($n = 40$) vs. IL6R 48892A/A ($n = 20$)]. C, patients treated with PPV were divided into four subgroups according to the IL6R 48892A>C polymorphisms (IL6R 48892C/C or A/C vs. IL6R 48892A/A) and IL6 levels (negative or positive) in prevaccination plasma [IL6 (−), IL6R C/C or A/C ($n = 18$); IL6 (−), IL6R A/A ($n = 11$); IL6 (+), IL6R C/C or A/C ($n = 21$); IL6 (+), IL6R A/A ($n = 9$)].

immune responses, we thought that this approach would be more rational than selecting nonpersonalized universal tumor antigens. Because tumor tissues were unavailable in most patients with aCRC, it was difficult to precisely characterize tumor cells in individual patients. Therefore, we selected and administered multiple (up to four) antigens to increase the possibility that the antigens used for vaccination were expressed in tumor cells.

We currently measure preexisting antigen-specific IgG responses, but not T-cell responses, for personalized selection of antigen peptides from a panel of candidate antigens, because antigen-specific T-cell assays often show limited sensitivity due to quite low frequencies of antigen-specific T cells before vaccinations, even after *in vitro* cell culture for expansion. Indeed, if the preexisting CTL responses in prevaccination PBMCs were used for selection of peptides in this study, much smaller numbers of peptides would be selected for vaccination (Supplementary Table S4). In contrast, the multiplex bead–based LUMINEX technology allows high-throughput screening of IgG responses specific to large numbers of peptide antigens with high accuracy (10, 11). Our previous studies suggested the clinical significance of antigen-specific IgG responses as a surrogate biomarker in monitoring vaccine-induced immune responses (14). In addition, this study demonstrated that increased IgG responses against administered peptides after vaccination were significantly associated with increased CTL responses. These results support our hypothesis that evaluation of IgG responses might be useful for predicting peptides that could induce specific CTL responses.

Because the vaccine peptides used for PPV are HLA-restricted CTL epitopes, they might act mainly through peptide-specific CTL responses. Indeed, peptide-specific CTL responses were significantly associated with OS (Table 4). Nevertheless, IgG responses to the vaccine peptides might also affect antitumor immunity. For example, in our preliminary study in mice, antibody complex with specific peptides facilitated the uptake of peptides and enhanced the cross-presentation of these peptides by antigen-presenting cells (S. Matsueda and colleagues; unpublished data). Further studies are currently in progress for clarification of the biologic functions of peptide-specific IgG.

Because not all patients show clinical benefits from cancer immunotherapies, it would be critical to identify prognostic or predictive biomarkers for patients receiving such therapies. Several postvaccination biomarkers have been reported to be associated with clinical responses (14–18), but there are currently no validated prevaccination predictive biomarkers. By multivariate analysis, higher IL6 and IP-10 and lower BAFF levels in prevaccination plasma were significantly associated

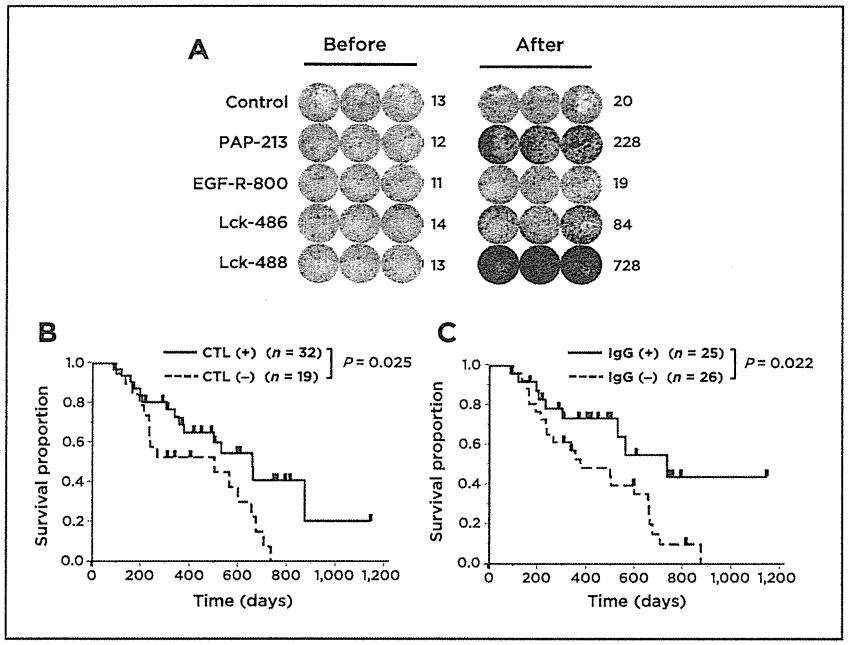

Figure 3. Prognostic significance of increased peptide-specific CTL or IgG responses in patients with aCRC after PPV. CTL and IgG responses specific to the vaccine peptides were determined by IFNγ ELISPOT and bead-based multiplex assays, respectively. A, a representative result of IFNγ ELISPOT assay with PBMCs stimulated with control (HIV-derived peptide) or vaccine peptides is shown before and after vaccination (Patient #4). Average spot numbers of triplicate wells are shown. B and C, patients treated with PPV were divided into two subgroups according to the presence or absence of increased peptide-specific CTL responses (B) or IgG responses (C) after the first cycle of vaccination. Curves for OS were estimated by the Kaplan–Meier method, and a difference between survival curves was statistically analyzed by the log-rank test. Censored patients are shown as vertical bars.

with unfavorable OS, although these factors might be prognostic irrespective of treatment, and not necessarily predictive and unique to PPV. Of note, however, the IP-10 level was predictive of the increase in CTL responses, which was associated with improved OS, suggesting that IP-10 might be potentially useful for selecting patients with aCRC, who would benefit from PPV. To more clearly assess the causal relation of IP-10, CTL responses, and OS, and to elucidate prognostic versus predictive relevance of such biomarkers, future randomized, controlled clinical trials with or without PPV would be essential.

IL6 has been reported to induce suppressive immune cell subsets, such as myeloid-derived suppressor cells and Th17 cells (19–22). Therefore, high levels of IL6 might inhibit immune responses to cancer vaccines by inducing these suppressive cells. BAFF is a cytokine for the differentiation and survival of follicular B cells along with humoral response potentiation (23). As previously suggested (24–26), BAFF might induce beneficial humoral immune responses to vaccine antigens. IP-10 is a chemokine for attraction of human monocytes, activated T cells, and NK cells (27, 28). Although local production of IP-10 within tumor tissues has been reported to be associated with antitumor immunity, systemic inflammatory responses mediated by IP-10 might contribute to poorer immune responses to vaccines (27, 28). The precise mechanisms of IL6, BAFF, and IP-10 in immune responses after PPV remain to be determined.

Results from this study suggested that the CRP 1846C>T and IL6R 48892A>C polymorphisms might show a statistically significant effect on OS after PPV. Because the CRP 1846C>T polymorphism, which affects serum CRP levels (29), has been reported to be associated with advanced diseases in patients with colorectal cancer (30) and

Table 3. Multivariate logistic regression analysis for predicting peptide-specific CTL or IgG responses after vaccination

	CTL responses		IgG responses	
Factor	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Number of previous chemotherapy regimens	0.996 (0.568–1.746)	0.989	1.012 (0.541–1.895)	0.970
Albumin, g/dL	0.640 (0.186–2.202)	0.479	2.847 (0.792–10.24)	0.109
CEA, ng/dL	0.772 (0.364–1.638)	0.501	1.008 (0.456–2.225)	0.985
IL6, pg/mL	0.565 (0.249–1.281)	0.172	0.685 (0.281–1.668)	0.404
IP-10, pg/mL	0.427 (0.191–0.957)	0.039	0.354 (0.127–0.982)	0.046
BAFF, pg/mL	0.885 (0.371–2.112)	0.783	1.205 (0.492–2.954)	0.683

Table 4. Multivariate Cox regression analysis for OS

	CTL responses		IgG responses	
Factor	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
CTL responses (positive vs. negative)	0.231 (0.067–0.803)	0.021	NA	NA
IgG responses (positive vs. negative)	NA	NA	0.790 (0.285–2.188)	0.650
Number of previous chemotherapy regimens	1.171 (0.777–1.765)	0.451	1.185 (0.776–1.808)	0.432
Albumin, g/dL	0.577 (0.297–1.124)	0.106	0.916 (0.510–1.645)	0.769
CEA, ng/dL	1.884 (1.115–3.183)	0.018	2.066 (1.204–3.544)	0.008
IL6, pg/mL	1.850 (1.101–3.107)	0.020	2.046 (1.220–3.432)	0.007
BAFF, pg/mL	0.400 (0.211–0.758)	0.005	0.578 (0.341–0.982)	0.043

Abbreviation: NA, not assessed.

esophageal squamous cell carcinoma (13), it might be a prognostic factor irrespective of the therapeutic approach. In contrast, because the IL6R 48892A>C polymorphism has been reported to show no effects on prognosis in some types of cancers, such as esophageal squamous cell carcinoma and neuroblastoma, without cancer vaccines (12, 31), the prognostic significance of this polymorphism might be unique to PPV-vaccinated patients. The IL6R 48892C (358Ala) allele has been reported to affect proteolytic cleavage of the membrane-bound IL6R, leading to reduced numbers of the functioning IL6R (32). As a result, this genetic variant is suggested to contribute to anti-inflammatory effect through attenuation of IL6 signaling on cells expressing the membrane-bound IL6R (33–35). On the basis of our finding, the effect of reduced IL6R expression might be more prominent when the availability of IL6 is limited, whereas it might be overcome by overexpression of IL6.

Importantly, this study demonstrated that successful boosting of peptide-specific CTL responses was significantly predictive of favorable OS by multivariate analysis, suggesting a causal relationship between biologic and clinical efficacy of PPV. However, peptide-specific IgG responses were not significantly predictive of OS by multivariate analysis, although they were significantly associated with favorable OS by the Kaplan–Meier method with the log-rank test. This discrepancy might be explained by the speculation that IgG responses might be more strongly affected by other confounding factors, such as IL6 and BAFF, compared with CTL responses. Because IL6 and BAFF are known to play important roles in the differentiation and survival of B cells along with humoral response potentiation (19, 23), it is possible that they substantially affected IgG responses, but not CTL responses, after vaccination.

In summary, this study demonstrated that PPV-induced substantial immune responses to vaccine antigens without severe adverse events and showed potential clinical benefits in previously treated patients with aCRC, even in the refractory stage. Nevertheless, this study has several drawbacks. First, this is a small study with a limited number of patients, all of whom received PPV. Second, combined chemotherapies and/or targeted therapies during the vaccination period might affect the occurrence of immune responses and conclusion about the prognostic versus the predictive role of biomarkers. Therefore, clinical efficacy of PPV, as well as clinical utility of the identified biomarkers, in patients with aCRC remain to be confirmed in future larger scale, randomized trials of PPV without combined chemotherapies or targeted therapies.

Disclosure of Potential Conflicts of Interest

T. Nomura is an employee of the Kyowa Hakko Kirin Co., Ltd. A. Yamada is a board member for Green Peptide Co., Ltd. K. Itoh reports receiving commercial research support from Taiho Pharmaceutical Co., Ltd., for which he has also received speakers bureau honoraria and is a consultant/advisory board member; he also has ownership interest (including patents) in Green Peptide Co., Ltd. No potential conflicts of interest were disclosed by the other authors.

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