SART3 p56lck

Fig. 2. Expression of the vaccine antigens in the tumor from a small cell lung cancer (SCLC) patient undergoing surgery after personalized peptide vaccination (PPV) treatment. The vaccine antigens SART3 and p56lck were detected by immunohistochemistry (IHC) with the antibodies specific to these molecules in the tumor tissue from a patient undergoing surgery after PPV treatment (Patient No. 5). Both sections, ×200.
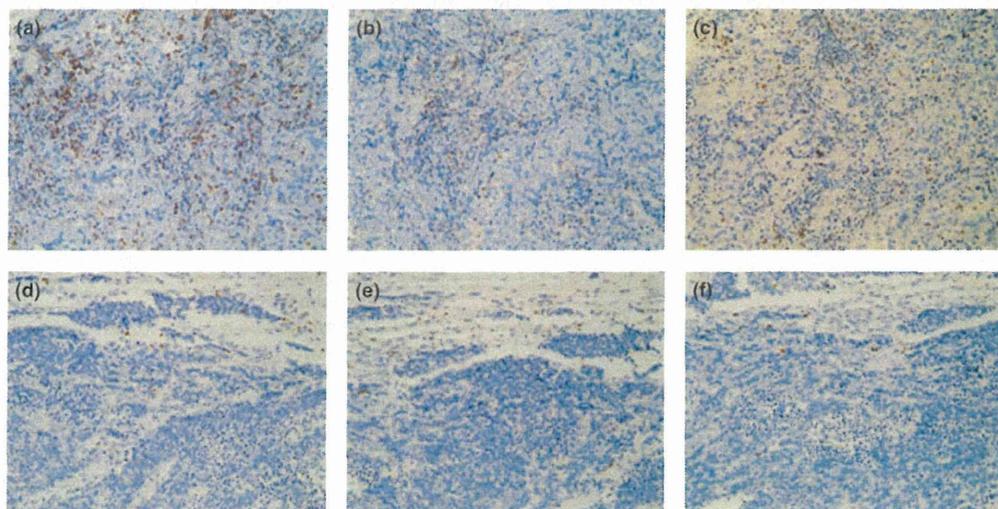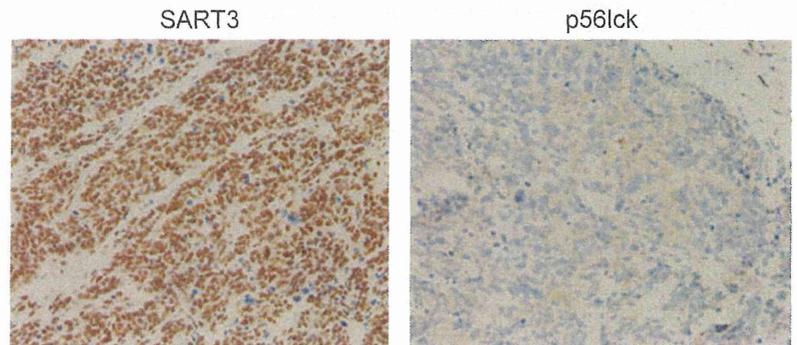

Fig. 3. Detection of tumor-infiltrating lymphocytes in tumors from small cell lung cancer (SCLC) patients treated with or without personalized peptide vaccination (PPV). Immune cells infiltrating within tumors were detected by immunohistochemistry (IHC) with the antibodies against CD3 (a and d), CD4 (b and e), and CD8 (c and f). All sections, ×100. (a–c) Tumor from a SCLC patient after PPV treatment (Patient No. 5). (d–f) Tumor from a SCLC patient without PPV treatment. Since the tumors from three SCLC patients without PPV treatment showed similar findings, representative data are shown.

limited number of patients developed a detectable Ab response, and there was no impact on clinical outcomes. In the current study, we addressed if refractory SCLC patients could have pre-existing IgG responses to 31 different vaccine candidates and well respond to these peptide vaccines. Notably, our results demonstrated that pre-vaccination plasma from all of the refractory SCLC patients had detectable levels of IgG specific to the cancer vaccine candidates, suggesting that they had the capability to show secondary immune responses to vaccine antigens. Furthermore, immunological boosting of T cell or IgG responses was observed in all of the patients, who completed at least one cycle of six vaccinations. Toxicity of PPV was mainly skin reactions at injection sites, and no SAE directly associated with the vaccinations were observed. These findings suggest the feasibility of PPV for refractory SCLC.

Interestingly, in a patient undergoing tumor resection after PPV, both CD4+ and CD8+ T cells infiltrated densely not only within the cancer stroma but also within the cancer cell nest. Since the vaccine antigens SART3 and p56lck were expressed in the tumor cells, it may be possible that T cells specific to these molecules infiltrated and accumulated within tumors. SART3 was strongly and homogeneously expressed in the tumor cells, whereas expression of p56lck was weak and heterogeneous. This heterogeneous expression of p56lck may be attributed to the immune escape mechanism of tumor cells

following PPV, although the pre-vaccination tumor tissue of this patient was unavailable to demonstrate this possibility.

The prognosis of refractory SCLC patients remains very poor with a median survival time of around 6–10 months.[1–3] Therefore, it could be worthwhile to discuss the clinical efficacy of PPV, although it was not the main objective of this study. In 10 refractory SCLC patients receiving PPV, the median OS was 186.5 days, with 1 year survival rate of 30%. In particular, six patients who received at least one cycle of six vaccinations survived for 25, 24.5 (alive), 10 (alive), 9.5, 6.5, and 6 months (median OS, 528 days), although survival time of the remaining four patients without completing six vaccinations was only 0.5, 0.5, 1, and 3 months (median OS, 25 days). Statistically analyses with clinical findings and laboratory data were performed to identify potentially prognostic factors, although the result was preliminary due to the small number of patients and its clinical utility needs to be confirmed in future studies. In the analysis of clinical findings, greater numbers of previous chemotherapy regimens might be associated with worse prognosis, suggesting that PPV should be considered before repeated failures of multiple chemotherapeutic regimens. Similar to our finding, the ability to mount an immune response to therapeutic vaccines was reported to be directly correlated with fewer prior chemotherapy regimens.[23] In addition, the statistical analysis with pre-vaccination laboratory data demonstrated that the frequency of CD3+CD26+

cells in PBMCs was potentially prognostic in patients receiving PPV. The frequency of CD3$^+$CD26$^+$ cells has not been previously reported as a biomarker in SCLC patients. CD26 is a cell surface glycoprotein that functions as a proteolytic enzyme, dipeptidyl peptidase IV (DPP IV), and has been reported to play a critical role in signal transduction.[24] Since this molecule is highly expressed on activated T cells,[24] the increased frequency of CD3$^+$CD26$^+$ might contribute to better immune responses against the vaccine antigens. The role of CD26$^+$ activated T cells in cancer vaccines remains to be determined.

In summary, the current study demonstrated that immune responses to the vaccine antigens were substantially induced without SAE in refractory SCLC patients who received at least one cycle (six times) of vaccinations. Nevertheless, due to the small number of patients and the short term of observation in this early phase trial, clinical efficacy of PPV for refractory SCLC remains to be confirmed in a next step of larger-scale, prospective trials.

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

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

Fig. S1. Immunohistochemical analysis of vaccine antigens in small cell lung cancer (SCLC) tissues.

Table S1. Peptide candidates for cancer vaccination.

Table S2. Frequency of expression of vaccine antigens in small cell lung cancer (SCLC) tissues.

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Personalized peptide vaccination in patients with refractory non-small cell lung cancer

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Abstract. Since the prognosis of non-small cell lung cancer (NSCLC) remains poor, the development of novel therapeutic approaches, including cancer vaccines, is highly desirable. In the current study, we conducted a phase II study of personalized peptide vaccination (PPV), in which a maximum of 4 peptides were selected based on pre-existing humoral immune responses and administered subcutaneously (weekly for 6 consecutive weeks and bi-weekly thereafter) in refractory NSCLC patients. Forty-one refractory NSCLC patients (4 stage IIIb, 22 stage IV and 15 recurrent), who had failed to respond to chemotherapy and/or targeted therapy (median number of regimens, 3; median duration, 10 months), were enrolled. Median overall survival (OS) was 304 days with a one-year survival rate of 42% in the enrolled patients. The main toxicity of PPV was skin reactions at the injection sites, but no serious adverse events were observed. In order to identify potential biomarkers for predicting OS, pre-vaccination and post-vaccination clinical findings and laboratory data were retrospectively assessed and evaluated by multivariate Cox regression analysis. Among the pre-vaccination factors examined, high C-reactive protein (CRP) level was a significant predictor of unfavorable OS [hazard ratio (HR)=10.115, 95% confidence interval (CI)=2.447-41.806, P=0.001]. Among the post-vaccination factors, high CRP level and low frequency of CD3+CD26+ cells were significant predictors of unfavorable OS (HR=23.127, 95% CI=2.919-183.233, P=0.003; HR=0.952, 95% CI=0.917-0.989, P=0.012). Taken together, our results suggest the feasibility of PPV for the treatment of refractory NSCLC. Evaluation of the identified factors before or at an early stage of vaccination could be potentially useful for selecting NSCLC patients who would likely have better prognosis following PPV.

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Introduction

Non-small cell lung cancer (NSCLC) is one of the most common causes of cancer death worldwide. Although recent advances in chemotherapy and/or targeted therapy have helped to improve the clinical outcomes of patients with refractory NSCLC (1-5), their prognosis still remains very poor with a median survival time of 6-8 months. Therefore, development of novel therapeutic approaches, including cancer vaccines, would be highly desirable.

We developed a new approach of peptide-based vaccination, named personalized peptide vaccination (PPV), in which vaccine antigens are selected and administered based on pre-existing host immunity before vaccination (6-14). We have shown promising results of PPV in various types of advanced cancers (6-9). For example, a recently conducted randomized clinical trial of PPV for patients with advanced prostate cancer suggested a potentially favorable clinical outcome in the vaccinated group (9). However, to improve clinical efficacy further, prognostic biomarkers that would make it possible to select patients for whom cancer vaccines would be appropriate remain to be identified. In the present investigation, we conducted a small-scale phase II study to identify potential biomarkers that would be useful for prediction of overall survival (OS) before or at an early stage of vaccination in refractory NSCLC patients. Our results suggested the feasibility of PPV for refractory NSCLC. The identified factors would be informative for predicting the subpopulation of NSCLC patients, who would likely have better prognosis following PPV.

Patients and methods

Patients. Patients with a histological diagnosis of NSCLC were eligible for inclusion in the present study, if they had failed to respond to previous chemotherapy and/or targeted therapy. They also had to show positive humoral responses to at least two of the 31 different candidate vaccine peptides (Table I), determined by both HLA class I type and the titer of IgG against each peptide. The other inclusion criteria, as well as the exclusion criteria, were not largely different from those of other previously reported clinical studies (6-9): patient age

between 20 and 80 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 1 or 2; positive status for HLA-A2, -A3, -A11, -A24, -A26, -A31, or -A33; life expectancy of at least 12 weeks; negative status for hepatitis virus B and C; adequate hematologic, renal, and hepatic function. Patients with lymphocyte counts of <1000 cells/μl were excluded from the study, since we had previously reported that pre-vaccination lymphopenia is a predictor of unfavorable OS in cancer patients receiving PPV (12). Other exclusion criteria included pulmonary, cardiac, or other systemic diseases; acute infection; a history of severe allergic reactions; pregnancy or nursing; or other inappropriate conditions for enrollment as judged by clinicians. The protocol was approved by the Kurume University Ethics Committee, and was registered in the UMIN Clinical Trials Registry (UMIN no. 1839). 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 primary and secondary endpoints were to identify potential biomarkers for OS and to evaluate the safety of PPV in NSCLC patients, respectively. Thirty-one peptides, whose safety and immunological effects had been confirmed in previously conducted clinical studies (6-9, 13), were employed for vaccination [12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for HLA-A3 supertype (-A3, -A11, -A31, and -A33), 4 peptides for HLA-A26] (Table I). The peptides were prepared under the conditions of good manufacturing practice (GMP) by the PolyPeptide Laboratories (San Diego, CA) and American Peptide Company (Vista, CA). Appropriate peptides for vaccination in individual patients were selected in consideration of pre-existing host immunity before vaccination, assessed from the titers of IgG specific to each of the 31 different vaccine candidates, as described previously (14). Combined chemotherapy and/or targeted therapy were allowed during the vaccination period, unless patients were unable to tolerate combined chemotherapies or declined them (Table II). A maximum of 4 peptides (3 mg/each peptide), which were selected on the basis of HLA typing and peptide-specific IgG titers, were administrated subcutaneously with incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France) once a week for 6 consecutive weeks. After the first cycle of 6 vaccinations, up to 4 antigen peptides, which were re-selected according to the titers of peptide-specific IgG in every cycle of 6 vaccinations, were administered every 2 weeks. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI-CTC Ver3). Complete blood counts and serum biochemistry tests were performed after every 6 vaccinations. The clinical responses were evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) in the vaccinated patients, for whom computed tomography (CT) scan or magnetic resonance imaging (MRI) data were available before and after the first cycle of vaccinations.

Measurement of humoral and T cell responses. The humoral responses specific to each of the 31 candidate peptides (Table I), including those employed and not employed for vaccination, were determined by the peptide-specific IgG levels using the Luminex system (Luminex, Austin, TX), as reported previously

(14). If the plasma titers of peptide-specific IgG in response to at least one of the vaccinated peptides after vaccination were >2-fold higher than those before vaccination, the changes were considered to be significant.

T cell responses specific to the vaccine peptides were evaluated by interferon (IFN)-γ Elispot using peripheral blood mononuclear cells (PBMCs), which were separated by density gradient centrifugation from peripheral blood (30 ml) with Ficoll-Paque Plus (GE Healthcare; Uppsala, Sweden) and stored frozen until analysis. After thawing, PBMCs (2.5×10^4 cells/well) were incubated in 384-well microculture plates (Iwaki, Tokyo, Japan) with 25 μl of medium (OpTmizer™ T Cell Expansion SFM; Invitrogen, Carlsbad, CA) containing 10% FBS (MP Biologicals, Solon, OH), IL-2 (20 IU/ml; AbD Serotec, Kidlington, UK), and each peptide (10 μM). Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) after culture for 3 days. After incubation for a further 6 days, the cells were harvested and tested for their ability to produce IFN-γ in response to either the corresponding peptides or a negative control peptide from human immunodeficiency virus (HIV) sequence (SLYNTYATL for HLA-A2; RYLRQQLLGI for HLA-A24; RLRDLLLIVTR for HLA-A3 supertype; EVIPMFSAL for HLA-A26). Antigen-specific IFN-γ secretion after 18 h of incubation was determined by Elispot, in accordance with the manufacturer's instructions (MBL, Nagoya, Japan). All assays were carried out in triplicate, and analyzed with the Zeiss Elispot reader (Carl Zeiss MicroImaging Japan, Tokyo, Japan). 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 peptide; a difference of at least 30 spots per 10^5 PBMCs was considered positive.

Measurement of C-reactive protein (CRP), serum amyloid A (SAA), and cytokines. CRP, SAA, and IL-6 in plasma were examined by ELISA using kits from R&D Systems (Minneapolis, MN), Invitrogen, and eBioscience (San Diego, CA), respectively. Multiplexed bead-based Luminex assays were used to measure Th1/Th2 cytokines, including IL-2, IL-4, IL-5, and IFN-γ (Invitrogen). Frozen plasma samples were thawed, diluted, and assayed in duplicate in accordance with the manufacturer's instructions.

Flow cytometric analysis of immune subsets among PBMCs. A suppressive immune subset, myeloid-derived suppressor cells (MDSCs), among PBMCs were examined by flow cytometry. For analysis of MDSCs, PBMCs (0.5×10^6) suspended in PBS containing 2% FBS were incubated with the following monoclonal antibodies (Abs) for 30 min at 4°C: anti-CD3-FITC, anti-CD56-FITC, anti-CD19-FITC, anti-CD33-APC, anti-HLA-DR-PE/Cy7, and anti-CD14-APC/Cy7. In the cell subset negative for lineage markers (CD3, CD19, CD56, CD14) and HLA-DR, MDSCs were identified as positive for CD33. The frequency of MDSCs in the lymphocyte gate defined by forward scatter and side scatter was calculated. In addition, the expression of CD26 in PBMCs was also analyzed, since the expression level of this gene assessed by cDNA microarray analysis has been shown to be predictive of OS in patients with prostate cancer receiving PPV (Sasada *et al*, unpublished data).

Table I. Peptide candidates for cancer vaccination.

Symbol for peptide	Protein	Position of peptide	Amino acid sequence	HLA type
CypB-129	Cyclophilin B	129-138	KLKHYGPGWV	A2, A3sup[a]
Lck-246	p56Lck	246-254	KLVERLGAA	A2
Lck-422	p56Lck	422-430	DVWSFGILL	A2, A3sup
MAP-432	ppMAPkkk	432-440	DLLSHAFFA	A2, A26
WHSC2-103	WHSC2	103-111	ASLDSDPWV	A2, A3sup, A26
HNRPL-501	HNRPL	501-510	NVLHFFNaPL	A2, A26
UBE-43	UBE2V	43-51	RLQEWCSVI	A2
UBE-85	UBE2V	85-93	LIADFLSGL	A2
WHSC2-141	WHSC2	141-149	ILGELREKV	A2
HNRPL-140	HNRPL	140-148	ALVEFEDVL	A2
SART3-302	SART3	302-310	LLQAEAPRL	A2
SART3-309	SART3	309-317	RLAEYQAYI	A2
SART2-93	SART2	93-101	DYSARWNEI	A24
SART3-109	SART3	109-118	VYDYNCHVDL	A24, A3sup, A26
Lck-208	p56Lck	208-216	HYTNaSDGL	A24
PAP-213	PAP	213-221	LYCESVHNF	A24
PSA-248	PSA	248-257	HYRKWIKDTI	A24
EGFR-800	EGF-R	800-809	DYVREHKDNI	A24
MRP3-503	MRP3	503-511	LYAWEPSFL	A24
MRP3-1293	MRP3	1293-1302	NYSVRYRPGL	A24
SART2-161	SART2	161-169	AYDFLYNYL	A24
Lck-486	p56Lck	486-494	TFDYLRSVL	A24
Lck-488	p56Lck	488-497	DYLRSVLEDF	A24
PSMA-624	PSMA	624-632	TYSVSFDSL	A24
EZH2-735	EZH2	735-743	KYVGIEREM	A24
PTHrP-102	PTHrP	102-111	RYLTQETNKV	A24
SART3-511	SART3	511-519	WLEYYNLER	A3sup
SART3-734	SART3	734-742	QIRPIFSNR	A3sup
Lck-90	p56Lck	90-99	ILEQSGEWWK	A3sup
Lck-449	p56Lck	449-458	VIQNLERGYR	A3sup
PAP-248	PAP	248-257	GIHKQKEKSR	A3sup

[a]A3sup, HLA-A3 supertype (A3, A11, A31, and A33).

PBMCs were stained with anti-CD26-PE and anti-CD3-FITC Abs. The frequency of the CD26^{+} subset among CD3^{+} cells was calculated. The samples were run on a FACSCanto II (BD Biosciences, San Diego, CA), and data were analyzed using the Diva software package (BD Biosciences). All Abs were purchased from Biolegend (San Diego, CA).

Statistical analysis. The two-sided Wilcoxon test was used to compare differences between pre- and post-vaccination measurements at a significance level of $P<0.05$. 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. The survival curve was estimated by the Kaplan-Meier method. Predictive factors for OS were evaluated by univariate and multivariate analyses with the Cox proportional hazards regression model. Statistically significant ($P<0.05$) variables in the univariate analysis were included in the multivariate analysis. Spearman rank correlation index was also utilized to choose the variables for multivariate analysis. All statistical analyses were conducted using the JMP version 8 or SAS version 9.1 software package (SAS Institute Inc., Cary, NC).

Results

Patient characteristics. Between December 2008 and October 2010, 41 patients with refractory NSCLC were enrolled in this study. Table II shows the clinicopathological characteristics of the enrolled patients. There were 19 male and 22 female subjects with a median age of 63 years, ranging from 37 to 76 years. Histologically, the tumors comprised 32 adenocarcinomas, 5 squamous cell carcinomas, 2 adenosquamous cell carcinomas, 1 large cell carcinoma, and 1 pleomorphic carcinoma. The

Table II. Characteristics of the enrolled patients with refractory NSCLC (n=41).

Patient no.	Histology	HLA type	Gender	Age	Stage	PS	Previous treatment No. of regimens	Previous treatment Period (months)	Combined therapy	No. of vaccination	Treatment response	OS (days)
1	Ad	A24	F	67	IV	0	1	2	CBDCA + PTX	24	SD	683
2	Ad	A26	F	56	R	0	5	16	S-1	24	SD	691
3	Ad	A11/A31	M	70	IV	0	1	5	-	6	PD	58
4	Ad	A24	F	69	IV	1	4	4	-	15	PD	225
5	Adsq	A2/A24	M	68	IIIb	0	3	5	Erlotinib	7	PD	95
6	Adsq	A24/A33	F	52	R	0	2	18	Erlotinib	6	NA	467
7	Ad	A2/A33	M	63	IV	0	1	1	-	4	NA	41
8	Ad	A2/A24	F	53	R	1	5	24	GEM	9	PD	159
9	Pleo	A24	M	55	R	0	2	6	DOC	3	NA	41
10	Ad	A2/A26	M	50	R	0	1	11	CBDCA + PTX	6	NA	422
11	Ad	A2/A24	M	57	IIIb	0	1	6	-	18	SD	354
12	Ad	A24	M	72	IV	0	1	4	-	22	SD	596[b]
13	Sq	A11/A33	F	53	IV	0	2	8	Gefitinib	6	SD	573[b]
14	Ad	A26	M	75	R	0	2	10	-	17	SD	366
15	Ad	A2	F	59	IV	0	3	10	Gefitinib	8	PD	291
16	Ad	A2	F	54	IV	1	4	24	CDDP + PEM	2	NA	304
17	Ad	A24	F	72	IV	0	1	25	-	11	SD	266
18	Ad	A2/A33	F	69	R	0	6	23	-	5	NA	51
19	Ad	A2/A31	F	76	R	0	3	4	-	6	NA	503[b]
20	Ad	A2/A11	M	61	IV	0	1	4	DOC	6	NA	431
21	Ad	A2/A11	F	65	R	0	1	3	Gefitinib	20[a]	SD	412[b]
22	Ad	A2/A11	M	50	IV	0	1	2	-	14	NA	356
23	Ad	A24/A33	M	67	R	0	3	9	-	17	SD	398[b]
24	Ad	A2/A3	M	70	IV	0	2	12	-	6	NA	230
25	Ad	A24/A33	F	68	IV	1	4	9	-	7	PD	81
26	Ad	A26/A33	F	65	IV	0	6	30	-	5	NA	208
27	Ad	A2/A26	F	70	IV	0	3	21	Erlotinib	11	SD	258
28	Ad	A24/A26	M	53	R	0	4	13	-	11	NA	189
29	Ad	A24	M	54	IV	0	5	13	-	8	PD	77
30	Ad	A24	M	37	R	0	2	10	PEM	14	PD	239[b]
31	Sq	A2/A24	M	64	IIIb	0	3	6	VNR	14[a]	NA	232[b]
32	Ad	A2/A24	F	59	R	0	3	43	Gefitinib	16[a]	SD	251[b]
33	Ad	A24	F	73	IIIb	0	10	72	-	11	PD	246[b]

Table II. Continued.

Patient no.	Histology	HLA type	Gender	Age	Stage	PS	Previous treatment No. of regimens	Previous treatment Period (months)	Combined therapy	No. of vaccination	Treatment response	OS (days)
34	Sq	A2/A24	F	62	IV	0	1	2	-	4	NA	50
35	Ad	A26/A33	F	54	IV	0	3	17	Gefitinib	14[a]	NA	239[b]
36	Sq	A24/A11	M	60	IV	0	3	12	-	15[a]	NA	237[b]
37	LCC	A24/A26	M	70	IV	0	6	19	-	14[a]	SD	190[b]
38	Sq	A2	M	66	R	1	3	6	-	10	PD	127
39	Ad	A2/A30	F	57	IV	0	4	32	PEM + Gefitinib	13[a]	PD	181[b]
40	Ad	A24/A26	F	44	R	0	3	23	Erlotinib	12	SD	176[b]
41	Ad	A2/A26	F	57	IV	0	2	11	-	12[a]	SD	176[b]

[a]Under treatment, [b]patients alive. NSCLC, non-small cell lung cancer; Ad, adenocarcinoma; Adsq, adenosquamous carcinoma; LCC, large cell carcinoma; Ple, Pleomorphic carcinoma; Sq, squamous cell carcinoma; M, male; F, female; R, recurrent; PS, performance status: CBDCA, carboplatin; PTX, paclitaxel; GEM, gemcitabine; DOC, docetaxel; CDDP, cisplatin; PEM, pemetrexed; VNR, vinorelbine; SD, stable disease; PD, progressive disease; NA, not assessed; OS, overall survival.

patients' cancers were at the refractory stage (stage IIIb, n=4; stage IV, n=22; recurrent, n=15) when they had failed to respond to one (n=11), two (n=7), three (n=11), or >4 (n=12) regimen(s) of chemotherapy, targeted therapy, and/or a combination of them. The median duration of these preceding regimens prior to PPV was 10 months, ranging from 1 to 72 months. Performance status at the time of enrollment was grade 0 (n=36) or grade 1 (n=5). The numbers of peptides used for vaccination of the patients during the first cycle were 4 peptides in 31 patients, 3 in 5 patients, and 2 in 5 patients. Among the 41 patients, 35 completed the first cycle of 6 vaccinations, whereas the remaining 6 patients failed to do so due to rapid disease progression. The median number of vaccinations was 11, with a range of 2 to 24. Among the 25 vaccinated patients for whom both pre- and post-vaccination radiological findings were available, none had a complete response (CR) or partial response (PR). The best response, seen in 14 patients, was stable disease (SD); the remaining 11 patients had progressive disease (PD).

Toxicities. Toxicities are shown in Table III. The most frequent adverse events were skin reactions at the injection sites (n=28) and hypoalbuminemia (n=21). One grade 4 serious adverse event (SAE), anemia, was noted. Grade 3 SAEs comprised injection site reaction (n=2), fever (n=1), hemoptysis (n=1), anemia (n=1), lymphopenia (n=1), and thrombocytopenia (n=1). According to evaluation by the independent safety evaluation committee for this trial, all of these SAEs, except for two cases of grade 3 injection site reaction, were concluded to be not directly associated with the vaccinations, but with cancer progression or other causes.

Immune responses to the vaccine peptides. Both humoral and T cell responses specific to the vaccine peptides were analyzed using blood samples obtained before and after the PPV. Plasma samples were obtained from 41, 35 and 18 patients before vaccination and at the end of the first (6 vaccinations) and second (12 vaccinations) cycles, respectively. Due to disease progression, 6 patients failed to complete the first cycle of 6 vaccinations. For monitoring of humoral immune responses, peptide-specific IgG reactive with each of the 31 different peptides, including those employed and not employed for vaccination, were measured by bead-based multiplex assay. The IgG responses specific to at least one of the vaccine peptides were augmented in 17 of 35 patients (49%) and in all of the 18 patients (100%) examined at the end of the first and second cycles of vaccination, respectively (data not shown).

T cell responses to the vaccine peptides were measured by IFN-γ Elispot assay. PBMCs from 36, 32 and 9 patients were available for this assay before and at the end of the first (6 vaccinations) and second (12 vaccinations) cycles, respectively. In the pre-vaccination samples, antigen-specific T cell responses were detectable in only 8 patients (22%). Among the 32 patients at the end of the first cycle of vaccinations, 11 (34%) showed T cell responses to the vaccine peptides. Among the 9 samples at the end of the second cycle of vaccinations, T cell responses were observed in 5 patients (56%) (data not shown).

Collectively, an increase of peptide-specific IgG titers was observed in about half and in all of the vaccinated patients at the end of the first and second cycles, respectively. In contrast,

Table III. Toxicities.

Toxicity type	Grade 1	Grade 2	Grade 3	Grade 4
Skin reactions at injection sites (n=28)	10	16	2	0
Constitutional symptom				
Fever (n=3)	1	1	1	0
Pulmonary/upper respiratory				
Dyspnea (n=3)	1	2	0	0
Hemoptysis (n=1)	0	0	1	0
Blood/bone marrow				
Anemia (n=11)	9	0	1	1
Leukocytopenia (n=7)	5	2	0	0
Neutropenia (n=4)	3	1	0	0
Lymphopenia (n=12)	10	1	1	0
Thrombocytopenia (n=2)	1	0	1	0
Laboratory				
Hyperbilirubinemia (n=3)	1	2	0	0
AST elevation (n=3)	2	1	0	0
ALT elevation (n=4)	3	1	0	0
Hypoalbuminemia (n=21)	17	4	0	0
Creatinine elevation (n=1)	1	0	0	0
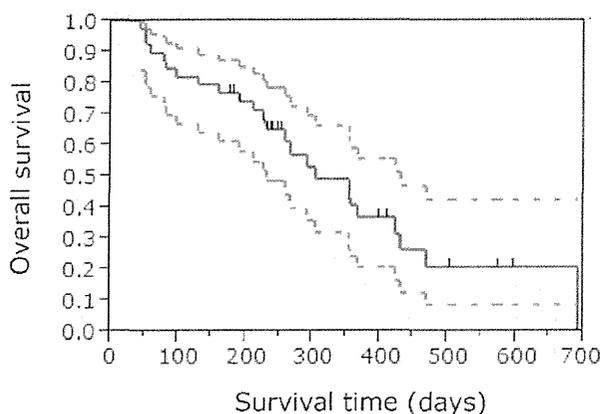
Figure 1. Kaplan-Meier survival analysis in the NSCLC patients receiving PPV. The median overall survival of patients who received PPV (n=41, solid line) was 304 days and the one-year survival rate was 42%. Dotted lines show 95% confidence intervals.

antigen-specific T cell responses were induced in only limited patients even after vaccination.

Cytokines and inflammation markers. We then measured cytokines (IL-2, IL-4, IL-5, IL-6, IFN-γ) and inflammation markers (CRP and SSA) in plasma before and at the end of the first cycle of vaccinations. IL-6 was detectable in 23 of 41 patients before vaccination, with a median level of 1 pg/ml, ranging from 0 to 103 pg/ml. Among the 35 plasma samples available at the end of the first cycle of vaccination, IL-6 levels were increased, decreased, and unchanged in 13, 7, and 15 patients, respectively. There was no significant difference in the

level of IL-6 before and after vaccination (P=0.614, Wilcoxon test). However, the 22 patients who showed a decrease or no change in IL-6 levels after vaccination had a tendency to have a better prognosis than the remaining 13 patients who showed an increase in IL-6 (P=0.068, log-rank test). Other cytokines, including IL-2, IL-4, IL-5, and IFN-γ, were rarely detectable in either pre- or post-vaccination plasma (data not shown).

The inflammation marker, CRP, was detectable in pre-vaccination plasma from the majority of patients (40 of the 41 patients), with a median level of 0.39 mg/dl (ranging from 0 to 1.11 mg/dl). Among the 35 plasma samples tested at the end of the first cycle of vaccination, plasma CRP levels were increased and decreased in 30 and 5 patients, respectively. Another inflammation marker, SAA, was also detected in pre-vaccination plasma from the majority of patients (40 of the 41 patients), with a median level of 6.21 mg/dl (ranging from 0 to 14.12 mg/dl). Among the 35 plasma samples available at the end of the first cycle of vaccination, plasma SAA levels were increased and decreased in 25 and 10 patients, respectively. There were significant increases in the levels of CRP (P<0.001, Wilcoxon test) as well as SAA (P=0.005, Wilcoxon test) after vaccination, compared with those before vaccination. However, there were no significant associations between changes in CRP or SAA levels and clinical outcomes in the vaccinated patients (data not shown).

Flow cytometric analysis of immune subsets among PBMCs. Immune cell subsets among both pre-vaccination and post-vaccination PBMCs were examined by flow cytometry. The median frequency of MDSCs among pre- and post-vaccination PBMCs was 0.4% (range, 0.1-3.4%, n=33) and 0.3% (range, 0.1-2.0%, n=33), respectively. There was a significant decrease in the frequencies of MDSCs after vaccination (P=0.002, Wilcoxon

Table IV. Univariate and multivariate analysis with pre-vaccination clinical findings or laboratory data.

Factor	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Age	1.006 (0.963-1.051)	0.786		
Gender	0.633 (0.281-1.428)	0.271		
Duration of previous treatment (months)	0.985 (0.934-1.039)	0.589		
Number of previous regimens	1.017 (0.807-1.282)	0.889		
Frequency of lymphocytes (%)	0.945 (0.898-0.993)	0.026		
Hemoglobin (g/dl)	0.826 (0.629-1.083)	0.167		
Albumin (g/dl)	0.220 (0.086-0.563)	0.002		
IL-6 (pg/ml)	1.021 (1.003-1.039)	0.020		
CRP (mg/dl)	9.375 (2.350-37.403)	0.002	10.115 (2.447-41.806)	0.001
Frequency of MDSCs (%)	1.089 (0.512-2.318)	0.825		
Frequency of CD3$^+$CD26$^+$ (%)	0.966 (0.914-1.021)	0.219		

CI, confidence interval; CRP, C-reactive protein; MDSCs, myeloid-derived suppressor cells.

Table V. Univariate and multivariate analysis with post-vaccination clinical findings or laboratory data.

Factor	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Skin reactions at injection sites	0.861 (0.287-2.585)	0.789		
Increase in T cell responses	0.708 (0.227-2.203)	0.551		
Increase in humoral responses	1.042 (0.407-2.669)	0.932		
Frequency of lymphocytes (%)	0.953 (0.909-0.999)	0.048		
Hemoglobin (g/dl)	0.745 (0.546-1.017)	0.064		
Albumin (g/dl)	0.169 (0.064-0.445)	<0.001		
IL-6 (pg/ml)	1.055 (1.023-1.087)	<0.001		
CRP (mg/dl)	13.250 (2.095-83.794)	0.006	23.127 (2.919-183.233)	0.003
Frequency of MDSCs (%)	0.833 (0.183-3.785)	0.813		
Frequency of CD3$^+$CD26$^+$ (%)	0.956 (0.916-0.998)	0.042	0.952 (0.917-0.989)	0.012

CI, confidence interval; CRP, C-reactive protein; MDSCs, myeloid-derived suppressor cells.

test). The median frequency of CD3$^+$CD26$^+$ cells among pre- and post-vaccination PBMCs was 18.8% (range, 7.4-47.0%, n=35) and 18.3% (range, 3.3-61.8%, n=35), respectively. There was no significant difference in the frequencies of CD3$^+$CD26$^+$ cells (P=0.965, Wilcoxon test) before and after vaccination. There were no significant associations between changes in the frequencies of MDSCs or CD3$^+$CD26$^+$ cells and clinical outcomes in the vaccinated patients (data not shown).

Relationship between clinical findings or laboratory data and OS. The median OS for the 41 patients was 304 days, with a one-year survival rate of 42% (Fig. 1). The Cox proportional hazards model was used to identify factors that were significantly associated with OS from clinical findings or laboratory data before vaccination. Univariate analysis using pre-vaccination data showed that albumin, CRP, SAA, IL-6, and the frequency

of lymphocytes in whole blood (P=0.002, P=0.002, P=0.004, P=0.020, and P=0.026, respectively) were significantly predictive of OS (Table IV). However, none of other factors examined, including age, gender, performance status, duration of chemotherapy or target therapy before vaccination, number of previous regimens, or other laboratory data (hemoglobin, creatinine, frequencies of regulatory T cells, MDSCs, or CD3$^+$CD26$^+$ cells), were significantly correlated with OS (data not shown). In addition, 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<0.05), after adjusting for possible confounding factors. Albumin, CRP, IL-6, and the frequency of lymphocytes in whole blood were included in the multivariate Cox regression analysis. SAA was excluded in this analysis, since the level of SAA was highly correlated with that of CRP (Spearman rank

correlation coefficient, 0.819; P<0.001). As shown in Table IV, higher CRP level in pre-vaccination plasma was significantly predictive of unfavorable OS [hazard ratio (HR)=10.115, 95% confidence interval (CI)=2.447-41.806, P=0.001]. However, the other factors showed no significant association.

Similarly, the Cox proportional hazards model was used to identify factors associated with OS from clinical findings or laboratory data at the end of the first cycle of vaccination. Univariate analysis showed that albumin, IL-6, SAA, CRP, frequency of CD3⁺CD26⁺ cells, and frequency of lymphocytes in whole blood were predictive of OS at the end of the first cycle of vaccination (P<0.001, P<0.001, P=0.004, P=0.006, P=0.042, and P=0.048, respectively) (Table V). None of the other factors, including other laboratory data, increase in IgG or T cell responses to the vaccine peptides, and skin reactions at the injection sites, were significantly correlated with OS. Albumin, IL-6, CRP, frequency of CD3⁺CD26⁺ cells, and frequency of lymphocytes were included in the multivariate Cox regression analysis. SAA was excluded in this analysis, since the level of SAA was highly correlated with that of CRP (Spearman rank correlation coefficient, 0.698; P<0.001). Multivariate Cox regression analysis demonstrated that higher CRP level and lower frequency of CD3⁺CD26⁺ cells in post-vaccination samples were predictive of unfavorable OS (HR=23.127, 95% CI=2.919-183.233, P=0.003; HR=0.952, 95% CI=0.917-0.989, P=0.012) (Table V).

Discussion

Since only a subset of patients obtain clinical benefits from peptide-based cancer vaccines, it would be critical to identify biomarkers for selection of suitable patients (15-17). With regard to post-vaccination biomarkers, we have shown that an increase in peptide-specific IgG responses after PPV is well associated with improved OS in patients with certain types of cancers (12,18). In addition, several factors, including cytotoxic T lymphocytes (CTL) responses, Th1 responses, delayed type hypersensitivity (DTH), and autoimmunity, have also been reported to be associated with clinical responses in some clinical trials (16,17,19,20), although these results have not always been reproducible. Notably, there are currently no validated pre-vaccination biomarkers, predictive of clinical responses, in widespread use. Therefore, in the present study, we searched for clinically useful predictive markers for PPV in patients with NSCLC. Multivariate analysis of pre-vaccination factors showed that higher level of plasma CRP was predictive of unfavorable OS. Among post-vaccination factors, higher level of plasma CRP and lower frequency of CD3⁺CD26⁺ cells were predictive of unfavorable OS. Although more data are still needed to validate our findings, evaluation of the factors identified here could be useful for selecting patients with NSCLC who would potentially benefit from cancer vaccines.

Elevated CRP level was shown to be also a predictor of unfavorable OS in NSCLC patients receiving chemotherapy or targeted therapy (21,22), suggesting that it might not necessarily be unique to vaccinated patients. In contrast, the frequency of CD3⁺CD26⁺ cells among PBMCs has not been reported previously as a biomarker in NSCLC patients. CD26 is a cell surface glycoprotein that functions as a proteolytic

enzyme, dipeptidyl peptidase IV, and plays a critical role in signal transduction (23). Since it is highly expressed on activated T cells (23), increased frequency of CD3⁺CD26⁺ might reflect the immune activation induced by vaccination. The role of CD26⁺ activated T cells induced by PPV in NSCLC thus remains to be determined.

MDSCs are a heterogeneous population of immature myeloid cells that inhibit the functions of other immune cells and promote tumor progression (24,25). MDSCs can facilitate tumor growth by inducing angiogenesis at tumor sites or by suppressing anti-tumor immune cells, such as antigen-specific T cells (24,25). Notably, the frequencies of MDSCs were significantly decreased after PPV. In addition, the patients who showed a decrease or no change in IL-6 after vaccination had a tendency to have better outcome. IL-6 is a multifunctional cytokine that regulates various aspects of cancer development, such as tumor cell growth and suppression of anti-tumor immune cells, including CTL and NK cells (26). The roles of these immune suppressive cells and/or cytokine, MDSCs and IL-6, in immune responses to cancer vaccines remain to be examined.

The prognosis of refractory NSCLC patients remains very poor, with a median survival time of 6-8 months (1-5). In contrast, the median OS of the 41 NSCLC patients who received PPV was 304 days (>10 months), with a one-year survival rate of 42%, in the current study. The main toxicity of PPV was skin reactions at the injection sites, but no SAEs were observed. Our previous trials of PPV for various types of cancers have also confirmed its safety (13). Considering the disease conditions of the patients enrolled in the current study, all of whom had already been resistant to or ineligible for conventional chemotherapeutic and targeted agents before enrollment, our findings suggest the feasibility of PPV for refractory NSCLC, even though OS was not the main objective of the current study. Nevertheless, since this is a retrospective study with a limited number of patients, clinical utility of PPV should be further verified in larger-scale, prospective trials conducted in defined patient populations with or without receiving PPV.

Acknowledgements

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Personalized peptide vaccination

A novel immunotherapeutic approach for advanced cancer

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

Introduction

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

Rationale for Personalized Selection of Vaccine Antigens in Individual Cancer Patients

A large number of tumor-associated antigens have been identified by several different approaches, including cDNA expression cloning, serologic analysis of recombinant cDNA expression libraries (SEREX), and reverse immunological approach.[8] Although the number of cancer vaccine candidates is becoming almost limitless, antigens currently employed
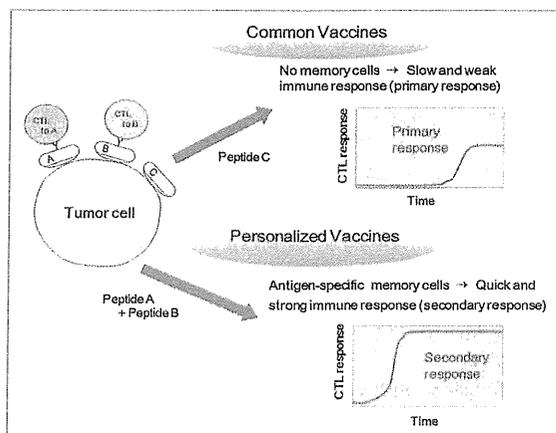
Figure 1. Personalized vaccines are more promising than common vaccines. Personalized antigens can induce quick and strong secondary immune responses, whereas common antigens without immunological memory induce slow and weak primary immune responses.

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

Nevertheless, in most of current clinical trials of therapeutic cancer vaccines, common antigens are employed for vaccination independently of immunological status of patients. Patients, who have immunological memory to vaccine antigens, are expected to show quick and strong immune responses to them. In contrast, patients with no immunological memory against vaccine antigens would take more time for development of effective anti-tumor immune responses, because several rounds of repeated vaccinations might be required to prime antigen-specific naïve T cells to functional effector cells (**Fig. 1**). In such situations, vaccinations could not

easily provide clinical benefits, especially in advanced cancer patients, who show a relatively quick disease progression. Moreover, immune responses induced by inadequate vaccines that are non-specific to tumor cells may not only be ineffective for tumor control, but also erode pre-existing immunity.[9] Based on the current paradigm that the size and composition of the adaptive immune system are limited and that individual immune cells are constantly competing each other in the limited space, inadequate vaccination may have negative consequences for the hosts by suppressing pre-existing beneficial memory cells specific to tumors and/or infections, which might result in acceleration of cancer progression or early death in vaccinated patients.[10] Considering these issues, it would be quite reasonable that vaccine antigens should be selected based on the pre-existing immunological status in each patient.

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

target multiple tumor-associated antigens to reduce the risk of outgrowth of antigen-loss variants.

PPV as a Novel Immunotherapeutic Approach

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

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

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

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

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

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

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

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

recurrent or progressive glioblastoma multiforme, one of the most aggressive brain tumors, with median overall survival of 10.6 mo.[19] Based on these promising results, randomized phase III trials are currently underway in CRPC and glioblastoma. To prove clinical benefits of PPV for accelerating cancer vaccine development, further randomized phase III trials would also be recommended in other different types of cancers.
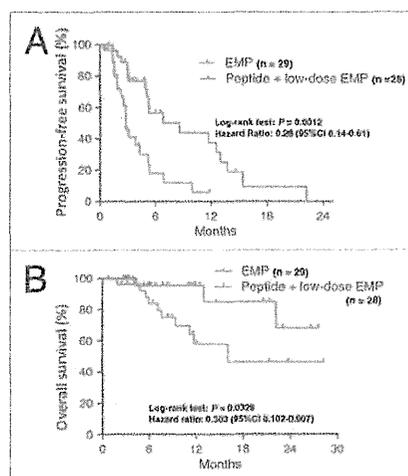
Lymphocyte Counts, Increased Humoral Responses to the Vaccine Antigens, and Inflammatory Factors as a Biomarker for PPV

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

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

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

To identify biomarkers useful for selecting appropriate patients before vaccination, we further addressed pre-vaccination prognostic markers in patients with several different types of advanced cancers who underwent PPV. In CRPC treated with PPV (n = 40), a comprehensive study of soluble factors and gene expression profiles by microarray analysis demonstrated that higher IL-6 level and granulocytic myeloid-derived suppressor cells (MDSC) in the peripheral blood before vaccination were closely associated with poorer prognosis.[24] In patients with refractory non-small cell lung cancer (n = 41), multivariate Cox regression analyses showed that higher C-reactive protein (CRP) level before vaccination was a significant predictor of unfavorable overall survival (HR = 10.115, 95% CI = 2.447–41.806, p = 0.001).[25] In addition, in refractory biliary tract cancer patients (n = 25), higher IL-6 and lower albumin levels before vaccination were significantly unfavorable factors for overall survival [HR = 1.123, 95% CI = 1.008–1.252, p = 0.035; HR = 0.158, 95% CI = 0.029–0.860, p = 0.033; respectively].[26] Collectively, these findings have demonstrated that less inflammation

may contribute to better responses to PPV, suggesting that evaluation of the inflammatory factors before vaccination could be useful for selecting appropriate cancer patients for PPV. Based on these findings, an early phase clinical trial is currently underway to show whether the blockage of IL-6-mediated inflammatory signaling with a humanized anti-IL-6 receptor monoclonal antibody, tocilizumab, would be beneficial for enhancing the immune and/or clinical responses of PPV.[27]

Conclusions

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

Disclosure of Potential Conflicts of Interest

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

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Review Article

Next-generation peptide vaccines for advanced cancer

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Many clinical trials of peptide vaccines have been carried out since the first clinical trial of a melanoma antigen gene-1-derived peptide-based vaccine was reported in 1995. The earlier generations of peptide vaccines were composed of one to several human leukocyte antigen class I-restricted CTL-epitope peptides of a single human leukocyte antigen type. Currently, various types of next-generation peptide vaccines are under development. In this review, we focus on the clinical trials of the following categories of peptide vaccines mainly published from 2008 to 2012: (i) multivalent long peptide vaccines; (ii) multi-peptide vaccines consisting of CTL- and helper-epitopes; (iii) peptide cocktail vaccines; (iv) hybrid peptide vaccines; (v) personalized peptide vaccines; and (vi) peptide-pulsed dendritic cell vaccines. (*Cancer Sci* 2013; 104: 15–21)

A cDNA-expression cloning technique to identify genes and peptides of tumor-associated antigens was first reported by van der Bruggen *et al.* in 1991.[1] Subsequently, a technique using autologous antibodies was introduced for identification of genes and peptides recognized by the host immune system.[2] These advanced techniques have provided a large number of antigens and peptides applicable as cancer vaccines. Many clinical trials of peptide vaccines have been carried out since the first clinical trial of a melanoma antigen gene-1 (MAGE-1)-derived peptide-based vaccine was reported in 1996 by Hu *et al.*[3] The earlier generations of peptide vaccines were composed of one to several human leukocyte antigen (HLA)-class I-restricted peptides of a single HLA-type. The peptides were emulsified with Montanide ISA51, a clinical grade of Freund's incomplete adjuvant, or pulsed on antigen-presenting cells and used for vaccination. Various types of new generation peptide vaccines have since been developed (Figs 1,2). In this review, we discuss the recent clinical trials of the latest generation of peptide-based cancer vaccines mainly published from 2008 to 2012.

Multivalent long peptide vaccines

The classical types of peptide vaccines only contain one to several epitope peptides, which are recognized by CTLs or helper T cells. In contrast, the mother proteins of the peptide vaccines usually contain several HLA-type restricted epitopes recognized by both CTLs and helper T cells. Although the importance of helper T cells in the induction of CTLs has been established and protein vaccines are able to induce both CTLs and helper T cells, the protein vaccines have several demerits in terms of manufacturing and safety controls. To avoid these drawbacks, synthetic long peptide vaccines have been developed. Synthetic long peptide vaccines are predominantly taken up by antigen presenting cells (APCs), where they are processed for presentation by both MHC class I and II molecules.

Several clinical studies using mixes of synthetic long peptides have been reported, as mixes of synthetic long peptide are likely to contain multiple HLA class I and II T-cell epitopes, which allows the use of this type of peptide vaccine in all patients irrespective of the type of HLA of each patient. Kenter *et al.*[4] carried out a phase I study of high-risk type human papilloma virus (HPV) 16 E6 and E7 overlapping long peptides in end-stage cervical cancer patients. Cocktails of nine E6 peptides and/or four E7 peptides, each 25–35-mer, covering the entire sequences of E6 and E7 proteins, were given s.c. with Montanide ISA51 four times at 3-week intervals. Co-injection of E6 and E7 long peptides induced a strong and broad T-cell response dominated by immunity against E6. Subsequently, they carried out a phase II study of this vaccine in patients with HPV-positive grade 3 vulvar intraepithelial neoplasia.[5] Vulvar intraepithelial neoplasia is a chronic disorder caused by HPV 16. At 3 months after the last vaccination, 12 of 20 patients (60%) had clinical responses and reported relief of symptoms. Five women had complete regression of the lesions. At 12 months of follow-up, 15 of 19 patients (79%) had clinical responses with a complete response in 9 of 19 patients (47%).

A synthetic long peptide vaccine targeted for p53 was reported by Speetjens *et al.*[6] The p53 synthetic long peptide vaccine consisted of 10 synthetic 25–30-mer long overlapping peptides, spanning amino acids 70–248 of the wild type p53 protein. Ten patients with metastatic colorectal cancer were vaccinated with this vaccine. The p53-specific T cell responses were induced in 9 of 10 patients as measured by γ-interferon (IFN-γ). Subsequently, a phase II study of a p53 synthetic long overlapping peptide vaccine in patients with ovarian cancer was carried out by the same group.[7] Twenty patients with recurrent elevation of CA-125 were immunized with the vaccine. Stable disease, as determined by CA-125 levels and computed tomography scans, was observed in 2/20 (10%) patients as the best clinical response, but no relationship was found with vaccine-induced immunity. Interferon-γ-producing p53-specific T-cell responses were induced in all patients who received all four immunizations. Interestingly, the IFN-γ secreted cells were CD4 T-cells and no CD8 T-cell/CTL responses were detected. The absence of CD8 T-cell/CTL responses may be attributable to the dominant production of

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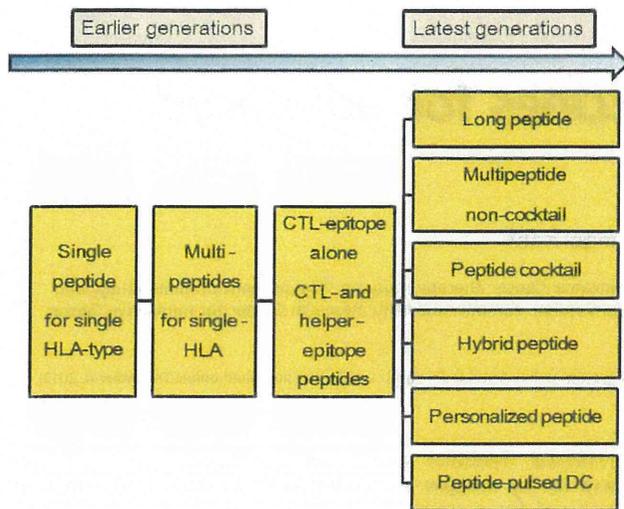E-mail: akiymd@med.kurume-u.ac.jp

Fig. 1. Transition of peptide vaccine development for advanced cancer. DC, dendritic cells.
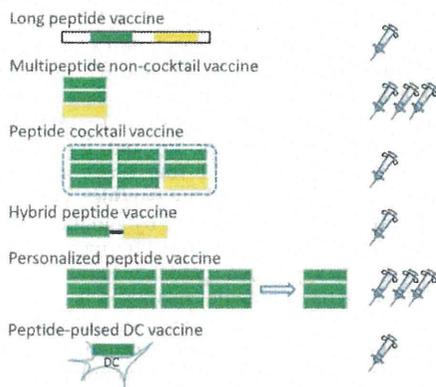

Fig. 2. Various types of latest generation peptide vaccines. The number of syringes indicates that of the final preparation for injection. Green, CTL-epitopes; orange, helper-epitopes. DC, dendritic cells.

Th2 cytokines, whose inhibitory effects on CTL induction are well known, although the vaccine immunization resulted in the expansion of p53-specific Th1 and Th2 CD4 T-cell responses.

Kakimi et al.[8] carried out a phase I trial of an NY-ESO-1 synthetic long peptide vaccine. A 20-mer NY-ESO-1f peptide, which includes multiple epitopes recognized by antibodies, and CD4 and CD8 cells, was given along with OK-432 and Montanide ISA51 to patients with advanced cancers. Both CD4 and CD8 T cell responses, as well as NY-ESO-1 antibody, were increased or induced in 9 of 10 patients.

Multipeptide vaccines consisting of CTL- and helper-epitopes

As mentioned above, helper T cells play crucial roles in the induction of CTLs. Some of the latest generation of peptide vaccines consist of HLA class-II restricted helper epitope peptides recognized by CD4 T cells in addition to class-I restricted CTL-epitope peptides to induce both CTLs and helper T cells. Numerous helper epitopes had been identified from the same target molecules of CTL-epitope vaccines and co-used as cancer vaccines.[9–17] A helper epitope peptide
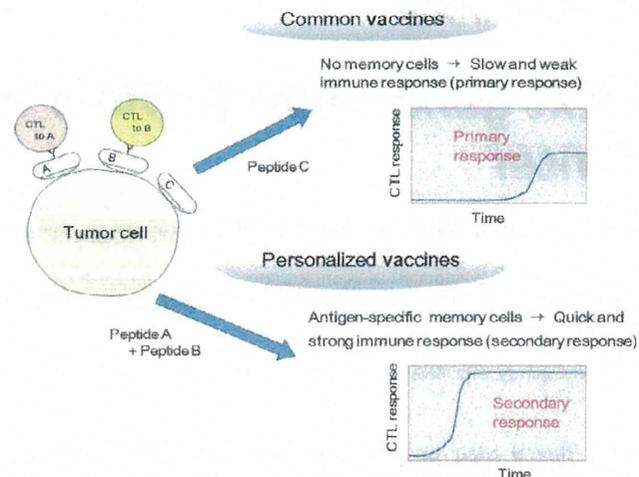

Fig. 3. Personalized peptide vaccine. In the classical type of vaccine, peptides derived from tumor-specific or overexpressed antigens are used as vaccine peptides and often mismatched to the pre-existing immunity of patients. In personalized peptide vaccines, appropriate peptides for vaccination are screened and selected from a panel of vaccine candidates in each patient, based on pre-existing host immunity and HLA types.

capable of binding pan HLA-DR (pan-DR epitope [PADRE]) has been reported,[18] and a clinical trial of a peptide vaccine using this helper epitope was reported. Kuball et al.[15] carried out a phase I study of CTL-epitope peptides of Wilms' tumor gene, proteinase 3, and mucin 1, and PADRE or mucin 1-helper epitope peptide with Montanide ISA51 and CpG oligonucleotide. Each peptide was formulated independently of the others and injected at a separate site. An increase in PADRE-specific CD4 T cells was observed after vaccination but these appeared unable to produce interleukin 2 (IL2), and the regulatory T cells were increased. This study indicates that helper epitope peptides have the potential to induce both helper T cells and regulatory T cells.

Peptide cocktail vaccines

Different peptides have different binding affinities to the corresponding HLA molecules. Therefore, if different CTL-epitope peptides with different binding affinities are loaded to APCs, there may be competition among the individual peptides to bind HLA molecules on the APCs. To prevent this, individual peptides of multipeptide vaccines were formulated independently of each other and injected at separate sites in most of the former clinical trials. In our case, a maximum of four peptides were individually mixed with Montanide ISA51 and injected s.c. at different sites on the same day. The maximum number of four peptides was similar to the maximum acceptable number of doses for patients on the same day, and no more than five peptides were used for vaccination. One of the strategies for overcoming the limitation of peptide number is the use of multipeptide cocktail vaccines. The multipeptide cocktail vaccines have no limitation of peptide number, as one preparation can contain more than 10 peptides. However, the issue of competition between the individual peptides of a cocktail vaccine for the binding of HLA molecules on the APCs still remains.

Different types of multipeptide cocktail vaccines have been developed, that is, vaccines consisting of CTL-epitope peptides alone,[19–21] or CTL-epitope and helper-epitope peptides.[9–13,16,17] The number of component peptides in the cocktail vaccines varies from around four to more than 10. Barve

Table 1. Immunological and clinical responses to personalized peptide vaccines for advanced cancer

Disease status	Phase	HLA restriction	Total no. of patients	Humoral response (%)	Cellular response (%)	Clinical response (%)	MST (months)	Grade 3/4 toxicities	Ref. no.
Advanced CRPC	PI	A24	10	60	40	SD 50	Not ref.	0	31
Advanced CRPC	PI	A24	13	91	55	PR 63	24	G3, 5%	32
Advanced CRPC	PI	A2	10	70	40	SD 30	22	0	33
Advanced CRPC	PI/II	A24	16	50	71	PR 43	17	0	37
Advanced CRPC	PI/II	A2/A24	58	88	78	PR 24	17	G3, 7%	38
Localized PC	PII	A24	10	80	80	PR 20	Not ref.	0	39
Advanced CRPC	PI, extension	A24	15	47	67	PR 13	24	0	46
Advanced CRPC	PII, randomized	A2/A24	57	64	50	PFS 8.5 (vaccine) vs 2.8M (control)	22.4 (vaccine) vs 16.1M (control)	0	44
Advanced CRPC	PII	A2/A24/ A3sup/A26	42	44	34	PR 12	17.8	0	49
Advanced malignant glioma	PI	A2/A24	21	40–64	50–82	PR 24, SD 38	Not reached	0	36
Advanced glioblastoma multiforme	PI, extension	A24	12	17	75	PR 17, SD 42	10.6	0	47
Advanced corolectal cancer	PI	A24	10	70	50	PR 10	Not ref.	0	34
Advanced corolectal cancer	PI/II	A2/A24	7	71	57	SD 14	Not ref.	G3, 20%	40
Advanced pancreatic cancer	PI	A2/A24	13	69	69	PR 15, SD 54	7.6	0	41
Non-resectable pancreatic cancer	PII	A2/A24	21	72	78	PR 33, SD 43	9	0	45
Advanced gastric cancer	PI	A2/A24	13	80	50	SD 45	Not ref.	0	30
Advanced lung cancer	PI	A24	10	40	40	SD 80	15.2	0	29
Refractory SCLC	PII	A2/A24/ A3sup/A26	10	83	83	SD 20	6.2	G3, 4%	50
Refractory NSCLC	PII	A2/A24/ A3sup/A26	41	49	34	SD 56	10.1	G3, 7%	42
Metastatic RCC	PI	A2/A24	10	80	5	SD 60	23	0	43
Malignant melanoma	PI	A2/A24	7	57	86	SD 43	Not ref.	0	28
Recurrent gynecologic cancer	PI	A2/A24	14	86	85	SD 36	Not ref.	G3, 8%	35
Advanced urotherial cancer	PI	A2/A24	10	80	80	CR 10, PR 10	24	0	48

A3sup, A3 super type; CR, complete response; CRPC, castration-resistant prostate cancer; G3, grade 3; HLA, human leukocyte antigen; M, months; MST, median survival time; Not ref., not referred; NSCLC, non-small-cell lung cancer; PI, phase I clinical trial; PII, phase II clinical trial; PC, prostate cancer; PD, progressive disease; PFS, progression-free survival; PR, partial response; RCC, renal cell carcinoma; Ref., reference; SCLC, small-cell lung cancer; SD, stable disease.
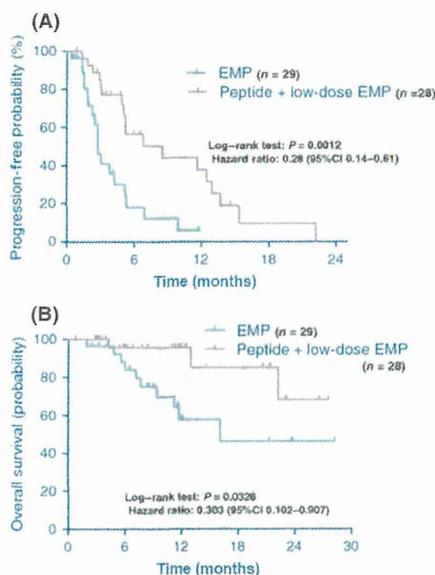
Fig. 4. Randomized phase II trial of personalized peptide vaccine (PPV) plus low-dose estramustine phosphate (EMP) versus standard-dose EMP in patients with castration-resistant prostate cancer. Patients were randomized into groups receiving either PPV plus low-dose EMP (280 mg/day) or standard-dose EMP (560 mg/day). (A) Duration of progression-free survival in the first treatment. (B) Overall survival of patients treated with PPV plus low-dose EMP and standard-dose EMP. CI, confidence interval.

et al.[9] carried out a phase I/II study of a cocktail vaccine IDM-2101 consisting of nine CTL-epitope peptides and the PADRE helper-epitope peptide with Montanide ISA51 in patients with metastatic non-small-cell lung cancer. No significant adverse events were noted except for low-grade erythema and pain at the injection site. One-year survival in the treated patients was 60%, and median overall survival was 17.3 months. One complete response case was observed in the total of 63 patients. Feyerabend and colleagues reported cocktail vaccines for patients with prostate cancer.[12] The cocktail vaccine consisted of 13 synthetic peptides, 11 HLA-A*0201 restricted CTL epitopes and two helper epitopes derived from prostate tumor antigens. A phase I/II trial of the vaccine was carried out in HLA-A2-positive patients with hormone-sensitive prostate cancer with biochemical recurrence after primary surgical treatment. The same group also developed another cocktail vaccine for renal cell cancer.[17] The vaccine, IMA901, consisted of nine HLA-A*0201 restricted CTL-epitopes and one helper epitope from renal cell cancer antigens with hepatitis B virus epitope as a marker peptide. A randomized phase II trial with a single dose of cyclophosphamide reduced the number of regulatory T cells and confirmed that immune responses to the vaccine component peptides were associated with longer overall survival.

Hybrid peptide vaccines

Peptide sequences of most of the single epitope vaccines as well as multi-epitope long peptide vaccines are native sequences with or without modification of anchor amino acids. Some of the latest generation of peptide vaccines are of hybrid-type, that is, a peptide fused with two epitopes. The Ii-Key/HER-2/neu hybrid peptide vaccine is a fusion peptide made up of the Ii-Key 4-mer peptide and human epidermal growth factor receptor-2 (HER-2)/neu (776–790) helper epitope peptide.[22,23] The Ii protein catalyzes direct charging

Table 2. Pros and cons of the latest generation of peptide vaccines

Vaccine type	Pros						Cons					
	Induction of CTL	Induction of Th	Applicable for multi-HLA type	Activation of memory T-cells	High efficiency of antigen presentation	Synthetic chemicals	No induction of Th	Possible induction of Treg	Not applicable for multi-HLA type	Multi formula	Induction of primary response	Biologics
Long peptide vaccine	Yes	Yes	Yes	No	No	Yes	No	Yes	No	No	Yes	No
Multipeptide non-cocktail vaccine	Yes	Yes	Yes	No	No	Yes	No	Yes	No	Yes	Yes	No
Peptide cocktail vaccine	Yes	Yes	Yes	No	No	Yes	No	Yes	No	No	Yes	No
Hybrid peptide vaccine	Yes	Yes		No	No	Yes	No	Yes	Yes	No	Yes	No
Personalized peptide vaccine	Yes	No	Yes	Yes	No	Yes	Yes	No	No	Yes	No	No
Peptide-pulsed DC vaccine	Yes	No	No	No	Yes	No	Yes/No	No	Yes	No	Yes	Yes

DC, dendritic cell; HLA, human leukocyte antigen; Th, helper T-cells; Treg, regulatory T-cells.