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ORIGINAL ARTICLE

Phase I trial of a cancer vaccine consisting of 20 mixed peptides in patients with castration-resistant prostate cancer: dose-related immune boosting and suppression

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Abstract The heterogeneity expression of tumor-associated antigens (TAA) and variability of human T cell repertoire suggest that effective cancer vaccine requires induction of a wide breadth of cytotoxic T lymphocyte (CTL) specificities. This can be achieved with vaccines targeting multiple TAA. We evaluated the safety and immune dynamics of a cancer vaccine consisting of 20 mixed peptides (KRM-20) designed to induce CTLs against 12 different TAA in patients with castration-resistant prostate cancer (CRPC). Patients received each of three different randomly assigned doses of KRM-20 (6, 20, or 60 mg) once a week for 6 weeks. KRM-20 was applicable for patients with positive human leukocyte antigen (HLA) A2, A3, A11, A24, A26, A31 or A33 alleles, which cover the majority of the global population. To evaluate the minimum immunological effective dose (MIED), peptide-specific CTL and immunoglobulin G (IgG) responses, and immune suppressive subsets were evaluated during the vaccination. Total of 17 patients was enrolled. No serious adverse drug reactions were encountered. The MIED of KRM-20 in CTL or IgG response calculated by logistic regression model was set as 16 or 1.6 mg, respectively. The frequency of immune suppressive subsets was fewer in the 20 mg cohort than that in 6 or 60 mg cohort. Clinical responses determined by prostate-specific antigen levels were two partial responses (from the 20 mg cohort), five no changes and ten progressive diseases. Twenty milligrams of KRM-20 could be recommended for further studies because of the safety and ability to augment CTL activity.

Keywords Cancer vaccine · Mixed peptide · Phase I · Prostate cancer · Immunotherapy

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Abbreviations

CR	Complete response
CRPC	Castration-resistant prostate cancer
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocyte antigen 4
ECOG	Eastern Cooperative Oncology Group
EGF-R	Epidermal growth factor receptor
HLA	Human leukocyte antigen
HNRPL	Heterogeneous nuclear ribonucleoprotein L
IgG	Immunoglobulin G
MDSC	Myeloid-derived suppressor cells
MRP3	Multidrug resistance-associated protein 3
PAP	Prostatic acid phosphatase
PBMC	Peripheral blood mononuclear cells
PD	Progressive disease
PFS	Progression-free survival
PPV	Personalized peptide vaccine
PR	Partial response
PSA	Prostate-specific antigen
PSMA	Prostate-specific membrane antigen
PTHrP	Parathyroid hormone-related peptide
SART3	Squamous cell carcinoma antigens 3
TAA	Tumor-associated antigen
T_{reg}	Regulatory T cells
UBE2 V	Ubiquitin-conjugated enzyme variant Kua
WHSC2	Wolf–Hirschhorn syndrome critical region 2

Introduction

Although numerous clinical studies of peptide-based cancer vaccines have been conducted in the past two decades, no outcome sufficient for drug approval has been obtained [1–3]. The heterogeneity of tumor-associated antigens (TAA) and the diversity of both human leukocyte antigens (HLA) and T cell subsets could hamper the successful development of therapeutic peptide vaccines [4, 5].

To overcome these hurdles, we newly developed a cancer vaccine consisting of 20 mixed peptides (KRM-20) from 12 different TAA that is applicable for patients with many different HLA alleles. These 12 TAA are highly expressed in prostate cancer tissues (prostate-specific antigen [PSA] [6], prostatic acid phosphatase [PAP] [7], prostate-specific membrane antigen [PSMA] [8], epidermal growth factor receptor [EGF-R] [9], parathyroid hormone-related peptide [PTHrP] [10]), and recognized by T cells and induced cancer-reactive CTLs (squamous cell carcinoma antigens [SART3] [11], cyclophilin B [12], Wolf–Hirschhorn syndrome critical region 2 [WHSC2] [13], UBE2V, ubiquitin-conjugated enzyme variant Kua [UBE2V] [13], heterogeneous nuclear ribonucleoprotein L [HNRPL] [13]) or having characteristics of T cell development and function ($p56^{lck}$ [14, 15], multidrug resistance-associated protein 3 [MRP3]) [15]. Twenty peptides used in this study were derived from these TAAs, and each peptide showed high immune responses to source TAA and clinical responses in previous clinical trials involving patients with CRPC [13]. All 20 peptides represent CTL epitopes, and these CTL epitopes are restricted by HLA-A2, A24, A3 supertype or A26 of major histocompatibility complex class I molecules, providing coverage of the vast majority of the general population. We also monitored regulatory T cells (T_{reg}), myeloid-derived suppressor cells (MDSC) and soluble cytotoxic T lymphocyte antigen 4 (sCTLA-4), three major types of suppressor cells for vaccine-induced CTL activity, to better understand immune dynamics during vaccination [16, 17]. We report here the results of a phase I, first-in-human study of this novel vaccine in patients with CRPC.

Patients and methods

Patient selection

Eligible patients had pathologically confirmed CRPC, age $\geq$ 20 years, Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1, positive status for HLA-A2, A24, A3 supertype (A3, A11, A31 and A33) or A26, life expectancy of at least 12 weeks, and adequate bone marrow function (white blood cell count $\geq$ 3,000/mm^3, lymphocyte count $\geq$ 1,000/mm^3, hemoglobin $\geq$ 8 g/dL, platelets $\geq$ 100,000/mm^3), hepatic function (total bilirubin $\leq$ 1.5 $\times$ the upper limit of normal [ULM]) and renal function (serum creatinine $\leq$ 1.5 $\times$ ULM). Patients were also required to have undergone bilateral orchiectomies or received ongoing treatment with a GnRH agonist or antagonist. Patients were required to have radiological progressive disease defined by computed tomography (CT), magnetic resonance imaging (MRI) or bone scan using the RECIST criteria, and/or prostate-specific antigen (PSA) progression defined as at least two consecutive rises and a level of $\geq$5 ng/ml for serum PSA taken over 2 weeks apart, in the setting of the castration level of testosterone ($\leq$50 ng/dl). Patients were excluded if they had had radiation therapy, chemotherapy, or immunotherapy within 4 weeks, immunosuppressive treatment using systemic steroid within 1 year except for using low-dose steroid (less than 30 mg/day prednisolone or less than 3 mg/day dexamethasone), multiple active cancers, a history of severe allergic reactions or severe symptoms caused by active infectious disease, circulatory disease, respiratory disease, kidney disease, immunodeficiency, or disturbance of coagulation. Patients who had received any of the peptides within the mixture of 20 peptides (KRM-20) were also excluded.

Study design

In this phase I, open-label, multicenter, randomized, dose-ranging, first-in-human study of KRM-20 as a single agent (Clinical trial registration, UMIN000008209), patients were randomized 1:1:1 to receive 6 mg/0.15 ml (0.3 mg/peptide), 20 mg/0.5 ml (1 mg/peptide), or 60 mg/1.5 ml (3 mg/peptide) of KRM-20 subcutaneously once a week for 6 weeks. These three dose levels were chosen based on previous clinical data, in which 3 mg/peptide was considered an acceptable dose according to its safety and immunological response [18, 19]. The sample size for each cohort was 5 for the completion of protocol treatment allowing adequate evaluation of safety and tolerability while minimizing exposure to a new cancer vaccine.

The primary objective was to evaluate the safety and immunological activity of different doses of KRM-20. The immunological activity was to determine the minimum immunological effective dose (MIED) of KRM-20 among three dose levels. The MIED was calculated by logistic regression model, and the study assumption is that MIED would be $\geq$60 % of immune responses. At each dose level, immunological response rates assessed by HLA-matched peptide-specific IgG and CTL levels at pretreatment, 3 weeks (1 week after the third vaccination) and 6 weeks (1 week after the sixth vaccination), were analyzed. Secondary and exploratory endpoints included relative change in serum PSA, T_{reg}, MDSC and CTLA-4.

The study was conducted according to the Declaration of Helsinki and relevant International Conference on Harmonization Good Clinical Practice guidelines, and was notified to a Japanese regulatory agency (Pharmaceuticals and Medical Devices Agency, PMDA) and the local institutional review boards of all four participating hospitals in Japan. All participants provided written informed consent before participating in this study.

Peptide preparation and preclinical study

KRM-20 consisted of the following 20 peptides: $SART3_{302-310}$, $Lck_{246-254}$, $UBE2V_{43-51}$, $WHSC2_{141-149}$ and $HNRPL_{140-148}$ for patients with HLA-A2; $Lck_{208-216}$, $Lck_{486-494}$, $Lck_{488-497}$, $MRP3_{1293-1302}$, $PAP_{213-221}$, $PSA_{248-257}$, $PSMA_{624-632}$, $EGF\text{-}R_{800-809}$ and $PTH\text{-}rP_{102-111}$ for patients with HLA-A24; $SART3_{734-742}$, Lck_{90-99} and $Lck_{449-458}$ for patients with HLA-A3 supertype; $SART3_{109-118}$ for patients with HLA-A24, A3 supertype or A26; $WHSC2_{103-111}$ for HLA-A2, A3 supertype or A26; and $CypB_{129-138}$ for patients with HLA-A2 or A3 supertype. Twenty peptides were prepared under conditions of current Good Manufacturing Practice using a Multiple Peptide System (San Diego, CA) and by the American Peptide Company (Vista, CA). The twenty peptides were mixed with incomplete Freund's adjuvant (Montanide ISA-51VG; Seppic, Paris, France) and prepared for emulsion at three different dose levels.

As KRM-20 is specific for the human host, the preclinical safety pharmacological study focused on possible effects of process-related impurities and related signs and symptoms of possible relevance for cardiovascular or respiratory in beagle, and central nervous system impairment in mice under conditions of Good Laboratory Practice (GLP). A 4-week subcutaneous subacute toxicity and 26-week chronic toxicity study in mice were also evaluated. In accordance with our previous observations [5–10], neither treatment-related deaths nor toxicologically important clinical or pathological signs were observed throughout these studies.

CTL and IgG responses

T cell responses specific to the vaccine peptides were evaluated by interferon (IFN)-γ Elispot assay (MBL, Nagoya, Japan), using peripheral blood mononuclear cells (PBMCs), which were separated by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare; Uppsala, Sweden) from peripheral blood (30 ml) before and after vaccination, and stored frozen until analysis. After thawing, PBMCs (1×10^5 cells/well) were incubated in 96-well U-bottomed plates (Thermo Fisher Scientific, Rochester, NY) with 100 µL of medium (OpTmizer™ T Cell Expansion SFM; Life Technologies, Carlsbad, CA) containing 10 % FBS (MP Biologicals, Solon, OH), 1 % L-glutamine (Life Technologies), IL-2 (20 IU/mL; AbD Serotec, Kidlington, UK) and a mixture of 20 vaccinated peptides (3 µg/mL each) for 6 days. The cultured cells were harvested and tested for their ability to produce IFN-γ in response to either the corresponding peptides or HLA-matched negative control peptides from human immunodeficiency virus (HIV) sequence (SLYNTYATL for HLA-A2; RYLRQQLLGI for HLA-A24; RLRDLLLIVTR for HLA-A3 supertype; EVIPMFSAL for HLA-A26). The cells (1×10^5 cells/well) were cultured in triplicate for 18 h at 37 °C with the C1R cells transfected with each type of HLA (1×10^4 cells/well) loaded with specific or control peptides (3 µg/mL) in a 96-well ELISPOT plate (MultiScreen, Millipore) coated with antihuman IFN-γ Ab. After washing, the spots were developed with biotin-conjugated antihuman IFN-γ Ab, streptavidin-ALP and BCIP/NBT substrate, according to the manufacturer's instructions (MBL), and then counted using an ELISPOT reader (ImmunoSpot S5 Versa Analyzer; Cellular Technology Ltd., Shaker Heights, OH). When the spot numbers in response to the specific peptides were significantly higher than those in response to the control peptides ($P < 0.05$ by Student's t test with the triplicate samples), antigen-specific CTL responses were shown

as the differences between them (means of the triplicate samples). If the spot numbers in response to at least one HLA-matched peptide per patient in the post-vaccination PBMC were more than twofold higher than those in the pre-vaccination PBMC, the changes were considered to be significant.

The humoral immune responses specific to the vaccine peptides were determined by peptide-specific IgG titers using a bead-based multiplex assay with the Luminex 200 system (Luminex, Austin, TX), as reported previously [20]. In brief, plasma before and after vaccination was incubated with 100 μL of peptide-coupled and peptide-uncoupled (negative control) color-coded beads for 1.5 h at 30 °C. After washing, the beads were incubated with 100 μL of biotinylated goat antihuman IgG Ab (gamma chain-specific; Vector Laboratories, Burlingame, CA) for 1 h at 30 °C. After washing again, the beads were incubated with 100 μL of streptavidin-PE (Life Technologies, Carlsbad, CA) for 30 min at 30 °C, followed by washing and detection of fluorescence intensity unit (FIU) on the beads using the Luminex 200 system. Peptide-specific IgG responses were defined by the difference between the FIU on peptide-coupled beads and that on peptide-uncoupled beads. If the titers of peptide-specific IgG in the post-vaccination plasma were more than twofold higher than those in the prevaccination plasma, the changes were considered to be significant. If a significant increase was observed in at least one vaccinated peptide, the specific humoral response was considered to be augmented.

T_{reg}, MDSC and sCTLA-4

T_{reg} and MDSC among the PBMC were examined by flow cytometry. For the analysis of T_{reg}, PBMC (0.5×10^6) suspended in PBS containing 2 % FBS were stained with anti-CD-4, anti-CD25 and anti-FoxP3 antibodies (Ab) by using the One Step Staining Human T_{reg} Flow™ Kit (Biolegend, San Diego, CA) in accordance with the manufacturer's instructions. For the analysis of MDSC, PBMC (0.5×10^6) suspended in PBS containing 2 % FBS were incubated with the following monoclonal Ab 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, MDSC were identified as positive for CD33. The samples were run on a FACSCanto II (BD Biosciences, San Diego, CA), and data were analyzed using the Diva software package (BD Biosciences). The frequencies of T_{reg} and MDSC in the lymphocyte gate defined by forward scatter and side scatter were calculated. All Ab were purchased from Biolegend. sCTLA-4 in plasma was analyzed using a commercially available kit (MedSystems Diagnostics, GmbH, Vienna, Austria).

Statistical analysis

The safety and tolerability analyses were performed on data from all patients receiving at least one dose of KRM-20. Immunological analyses were performed on data from all patients who completed the study. Because of the low planned number of patients in each cohort, descriptive statistics were used to summarize all demographic, safety, tolerability and immunological outcomes by dose cohort. Changes in immunological parameters across doses were assessed by an exploratory analysis of variance.

Results

Patient population

Between June 2012 and January 2013, 17 patients from four clinical sites in Japan were randomized to treatment with KRM-20 at three different doses: Arm A (6 mg/0.15 ml), $n = 5$; Arm B (20 mg/0.5 ml), $n = 6$; and Arm C (60 mg/1.5 ml), $n = 6$. Fifteen of these 17 patients completed the study as planned, and two patients discontinued treatment due to non-treatment-related serious adverse events of compression fracture of metastatic bone or urinary retention: one in the 20 mg dose cohort (Arm B) and the other in the 60 mg dose cohort (Arm C), respectively. Demographics and baseline characteristics were generally balanced among the treatment arms, although one patient had a high PSA level in Arm A (Table 1).

Safety

Adverse events were assessed using the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0 (NCI-CTC Ver. 4). There was no obvious difference in the rate of adverse events across the three dose cohorts. The most common treatment-related adverse event was skin reactions at injection sites (76.5 %). Two patients had a grade 2 increase of liver enzymes or herpes zoster. No grade 3/4 treatment-related adverse events were observed. All adverse events observed in this study are shown in Table 2.

Clinical outcomes and immune responses

All patients were assessed by CT of the abdomen and pelvis and bone scans within 4 weeks prior to the first vaccination of KRM-20, and serum PSA was evaluated at pretreatment, and after the third and sixth vaccination. Fifteen of seventeen patients completed the study treatment of six injections. According to the PSA response by the Prostate Cancer Clinical Trials Working Group (PCGW2) criteria [21], among these 15 patients, the best response was reported as a

Table 1 Patient baseline characteristics

	Arm A (6 mg/0.15 ml) ($n = 5$)	Arm B (20 mg/0.5 ml) ($n = 6$)	Arm C (60 mg/1.5 ml) ($n = 6$)	Total ($n = 17$)
Age, years				
Median	72	73.5	69	71.5
Range	60–77	68–77	55–76	55–77
ECOG performance status, *n*				
0	4	6	5	15
1	1	0	1	2
HLA typing, *n*				
A2/A3 supertype	1	2	2	5
A2/A26	0	0	1	1
A2	0	1	0	1
A24/A3 supertype	2	1	1	4
A24/A26	1	1	2	4
A24	1	0	0	1
A3 supertype	0	1	0	1
Baseline PSA, ng/ml				
Median	67.9	21.6	31.6	40.4
Range	1.02–1,529	5.23–84.8	8.72–81.47	1.02–1,529
Gleason score, *n*				
≤7	1	2	1	4
≥8	4	4	5	13
Site of metastasis, *n*				
No	0	0	1	1
Bone only	2	3	3	8
Bone and nodal/organ	1	3	2	6
Nodal / organ	2	0	0	2
Prior chemotherapy, *n*				
Estramustin phosphate	3	5	4	12
Docetaxel	2	2	3	7

ECOG Eastern Cooperative Oncology Group, *HLA* human leukocyte antigen, *PSA* prostate-specific antigen, HLA-A3 supertype including A11, A31 or A33

partial response (PR) in two patients in Arm B (20 mg dose cohort), no change (NC) in five and progressive disease (PD) in eight. Disease control (PR or NC) during the treatment was thus observed in 7 of 15 patients (Table 3).

Blood samples at pre-vaccination, and after the third and sixth vaccination in the 15 patients, were analyzed for HLA-matched peptide-specific IgG by LUMINEX and HLA-matched peptide-specific T cells by IFN-γ-based ELISPOT in a blinded fashion (Table 4). The median number of HLA-matched peptides was 10 (range 6–15) in all cases. In all HLA-matched peptides, CTL response after the third vaccination was boosted for 3 of 61, 12 of 45 and 11 of 50 peptides tested in patients receiving 6, 20 and 60 mg of KRM-20, respectively. CTL response after the sixth vaccination, however, largely decreased in patients receiving 60 mg, concomitant with strong boosting of the IgG response.

For each patient base after the sixth vaccination, CTL activity was augmented for at least one peptide in 1 of 5, 4 of 5 and 4 of 5 patients receiving 6, 20 and 60 mg of KRM-20, respectively. IgG level was also augmented in 4 of 5, 2 of 5 and all 5 patients, respectively. Collectively, 9 or 2 patients (two from the 60 mg cohort) showed boosted CTL or IgG responses after the third vaccination, while 9 or 11 patients showed this after the sixth vaccination, respectively.

Immune responses and PSA responses in 15 patients during the vaccination are shown in Fig. 1. Positive immune responses were observed in 13 of 15 patients (87 %), with 2 PR, 5 NC and 6 PD. In addition, CTL responses to two or more peptides (2–6 peptides), restricted by HLA-A2, A24, A3 supertypes or A26, were observed in 5 or 6 patients after the third or sixth vaccination.

T_{reg}, MDSC and sCTLA-4

The mean frequency of T_{reg} or MDSC among PBMC at pre-vaccination, and 3 and 6 weeks after the first vaccination were 2.0, 2.5 and 1.8 %, or 1.0, 1.2 and 1.4 %,

Table 2 Adverse events by treatment cohort and grade

	Arm A (6 mg/0.15 ml)			Arm B (20 mg/0.5 ml)			Arm C (60 mg/1.5 ml)			Total			
	$n = 5$			$n = 6$			$n = 6$			$n = 17$			
	G1	G2	G3	G1	G2	G3	G1	G2	G3	G1	G2	G3	All (%)
Any AEs													
Anemia			1									1	1 (5.9)
Toothache				1						1			1 (5.9)
Injection site skin reaction	3			5			5			13			13 (76.5)
Fever							1			1			1 (5.9)
Increased ALT and AST		1									1		1 (5.9)
Herpes zoster					1						1		1 (5.9)
Compression fracture of bone						1						1	1 (5.9)
Dehydration							1			1			1 (5.9)
Bone pain	1									1			1 (5.9)
Headache										1			1 (5.9)
Urinary retention									1			1	1 (5.9)
Microhematuria	1									1			1 (5.9)
Increased Creatinine				1						1			1 (5.9)
Treatment-related AEs													
Injection site skin reaction	3			5			5			13			13 (76.5)
Increased ALT and AST		1									1		1 (5.9)
Herpes zoster				1							1		1 (5.9)

AE adverse event, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *G* grade

Table 3 Changes of T_{reg}, MDSC and sCTLA-4, and PSA responses in 15 patients during vaccination

Patient No.	Treatment Arm	T_{reg} (%)			MDSC (%)			sCTLA-4 (ng/ml)			PSA response (% from base line)
		Pre	3rd	6th	Pre	3rd	6th	Pre	3rd	6th	
1	Arm A (6 mg/0.15 ml)	2.6	2.4	**5.7**	1.6	0.2	1.2	1.5	1.4	1.3	PD (63)
2		4.9	3.2	1.7	0.5	0.4	0.2	0.0	0.0	0.0	PD (45)
3		0.9	0.7	**1.3**	0.6	0.7	0.3	0.0	0.0	0.0	PD (182)
4		1.7	2.0	**2.8**	1.9	**4.8**	0.6	0.0	0.0	0.0	NC (24.8)
5		2.6	2.9	0.9	0.8	0.5	**2.3**	0.0	0.0	0.0	NC (−2.8)
6	Arm B (20 mg/0.5 ml)	3.3	3.4	2.5	0.6	0.8	1.3	0.0	0.0	0.0	NC (11)
7		1.0	0.9	0.7	0.2	0.1	**2.2**	0.3	0.3	0.3	PR (-65)
8		2.4	3.0	0.7	1.4	**4.1**	1.2	0.0	0.0	0.0	PD (40.8)
9		0.8	0.5	**1.8**	0.3	0.4	1.0	0.0	0.0	0.0	PD (102)
10		0.8	1.5	1.1	0.8	0.7	0.8	0.0	0.0	0.0	PR (-73)
11	Arm C (60 mg/1.5 ml)	1.2	1.6	1.3	1.6	1.8	1.2	0.2	0.1	0.2	NC (2)
12		2.4	**9.6**	2.7	0.6	0.3	0.6	0.0	0.0	0.0	PD (196)
13		2.0	**2.9**	1.2	0.1	0.3	**4.1**	1.7	1.6	1.9	NC (-39)
14		2.1	**2.7**	2.0	0.8	0.8	**2.8**	0.0	0.0	0.0	PD (886)
15		1.0	1.1	1.1	3.2	1.8	0.9	0.0	0.0	0.0	PD (206)

T_{reg} regularity T cell, *MDSC* myeloid-derived suppressor cell, *Pre* pretreatment, *sCTLA-4* soluble cytotoxic T lymphocyte antigen 4, *PSA* prostate-specific antigen, *PR* partial response, *NC* no change, *PD* progressive disease

The values showing the increment are in bold

respectively (Table 3). Although there was no significant difference among these mean frequencies, it is of note that the frequency of T_{reg} after the third or sixth vaccination was increased in 3 of 5 or 3 of 5 patients receiving 60 or 6 mg of KRM-20, respectively. In contrast, the increase after the third and sixth vaccinations occurred in one patient in the

Table 4 CTL and IgG responses to HLA-matched peptides in 15 patients during vaccination

Patient no. (HLA)	HLA matching peptides (Target HLA)	CTL response (spots/10^5× cells)			IgG Response (FIU)		
		Pre	3rd	6th	Pre	3rd	6th
Arm A (6 mg/0.15 ml)							
1 (A24, A11)	CypB-129 (A11)	35	0	0	0	0	0
	EGFR-800 (A24)	0	0	0	0	0	0
	Lck-208 (A24)	0	0	0	0	0	0
	Lck-449 (A11)	0	**20**	0	14	17	13
	Lck-90 (A11)	67	36	24	31	41	**1,805**
	MRP3-1293 (A24)	0	0	0	0	0	0
	PSA-248 (A24)	0	0	0	0	0	0
	PTHrP-102 (A24)	0	0	0	0	0	0
	SART3-734 (A11)	50	17	0	5,053	6,424	5,360
	WHSC2-103 (A11)	56	0	31	11	13	11
	Lck-486 (A24)	0	0	0	15	14	13
	Lck-488 (A24)	0	0	0	20	26	21
	PSMA-624 (A24)	0	0	0	0	0	0
	PAP-213 (A24)	0	0	0	10	13	**72**
	SART3-109 (A24, A11)	76	48	0	0	**12**	**12**
2 (A2, A31)	CypB-129 (A2, A31)	72	95	90	0	0	0
	Lck-246 (A2)	0	0	**55**	0	0	0
	Lck-449 (A31)	81	65	**227**	0	10	0
	Lck-90 (A31)	128	99	99	0	0	0
	SART3-302 (A2)	0	51	0	0	0	0
	SART3-734 (A31)	139	71	88	0	0	0
	WHSC2-103 (A2, A31)	96	117	**404**	0	0	0
	HNRPL-140 (A2)	0	**36**	0	0	0	0
	SART3-109 (A31)	76	56	72	0	0	0
	WHSC2-141 (A2)	0	0	0	0	0	0
	UBE2V-43 (A2)	0	0	**25**	0	11	**19**
3 (A24, A26)	EGFR-800 (A24)	0	0	0	14	16	13
	Lck-208 (A24)	0	0	0	0	0	0
	MRP3-1293 (A24)	0	0	0	19	18	17
	PSA-248 (A24)	0	0	0	0	0	0
	PTHrP-102 (A24)	0	0	0	0	0	0
	Lck-486 (A24)	0	0	0	35	39	33
	Lck-488 (A24)	0	0	0	140	149	142
	PSMA-624 (A24)	0	0	0	19	21	18
	PAP-213 (A24)	0	0	0	12	13	11
	SART3-109 (A24, A26)	0	0	0	53	57	49
4 (A24, A31)	CypB-129 (A31)	70	0	36	0	0	0
	EGFR-800 (A24)	0	0	0	10	10	11
	Lck-208 (A24)	0	0	0	0	0	0
	Lck-449 (A31)	121	86	103	24	20	21
	Lck-90 (A31)	153	0	97	24	21	21
	MRP3-1293 (A24)	0	0	0	12	11	12
	PSA-248 (A24)	0	0	0	11	0	0
	PTHrP-102 (A24)	0	0	0	0	0	0
	SART3-734 (A31)	140	74	0	13	10	10
	WHSC2-103 (A31)	56	0	31	11	10	10

Table 4 continued

Patient no. (HLA)	HLA matching peptides (Target HLA)	CTL response (spots/10^5× cells)			IgG Response (FIU)		
		Pre	3rd	6th	Pre	3rd	6th
	Lck-486 (A24)	0	0	0	21	19	19
	Lck-488 (A24)	0	0	0	32	29	30
	PSMA-624 (A24)	0	0	0	12	0	10
	PAP-213 (A24)	0	0	0	11	10	**4,139**
	SART3-109 (A24, A31)	113	**434**	141	22	22	21
5 (A24)	EGFR-800 (A24)	0	0	0	28	27	29
	Lck-208 (A24)	0	0	0	12	14	12
	MRP3-1293 (A24)	0	0	0	37	35	34
	PSA-248 (A24)	0	0	0	60	63	**819**
	PTHrP-102 (A24)	0	0	0	0	0	0
	Lck-486 (A24)	0	0	0	60	57	53
	Lck-488 (A24)	0	0	0	118	111	108
	PSMA-624 (A24)	0	0	0	37	38	33
	PAP-213 (A24)	0	0	0	42	38	37
	SART3-109 (A24)	0	0	0	58	57	54
Response rates (confidence intervals)		0.20 (0.0051–0.7164)			0.80 (0.2836–0.9949)		
Arm B (20 mg/0.5 ml)							
6 (A2, A33)	CypB-129 (A2, A33)	0	0	0	0	0	**17**
	Lck-246 (A2)	0	**451**	**472**	0	0	0
	Lck-449 (A33)	27	0	**130**	1,004	1,127	1,064
	Lck-90 (A33)	26	44	45	66	71	68
	SART3-302 (A2)	0	0	0	1,031	1,122	1,099
	SART3-734 (A33)	31	32	**62**	81	102	102
	WHSC2-103 (A2, A33)	48	46	30	18	20	19
	HNRPL-140 (A2)	0	0	**435**	38	45	45
	SART3-109 (A33)	25	30	35	33	35	35
	WHSC2-141 (A2)	0	0	0	12	12	11
	UBE2V-43 (A2)	0	0	0	15	14	15
7 (A2)	CypB-129 (A2)	0	**107**	0	0	0	0
	Lck-246 (A2)	0	**129**	**247**	0	0	0
	SART3-302 (A2)	0	**103**	0	16	20	18
	WHSC2-103 (A2)	0	**125**	0	16	17	14
	HNRPL-140 (A2)	0	**121**	0	15	17	15
	UBE2V-43 (A2)	0	0	0	32	33	27
	WHSC2-141 (A2)	0	**121**	0	0	0	0
8 (A24, A26)	EGFR-800 (A24)	0	0	0	0	0	0
	Lck-208 (A24)	0	0	0	0	0	0
	MRP3-1293 (A24)	0	0	0	18	19	19
	PSA-248 (A24)	0	0	0	0	0	0
	PTHrP-102 (A24)	0	0	0	0	0	0
	Lck-486 (A24)	0	0	0	27	27	26
	Lck-488 (A24)	0	0	0	41	39	38
	PSMA-624 (A24)	0	0	0	11	10	11
	PAP-213 (A24)	0	0	0	0	0	0
	SART3-109 (A24, A26)	0	0	0	14	14	12
9 (A2, A33)	CypB-129 (A2, A33)	0	**45**	0	0	0	0
	Lck-246 (A2)	49	**286**	**248**	0	0	0
	Lck-449 (A33)	72	104	0	13	11	11

Table 4 continued

Patient no. (HLA)	HLA matching peptides (Target HLA)	CTL response (spots/10^5× cells)			IgG Response (FIU)		
		Pre	3rd	6th	Pre	3rd	6th
	Lck-90 (A33)	108	131	140	21	16	40
	SART3-302 (A2)	0	0	**136**	2,765	2,099	2,824
	SART3-734 (A33)	112	**259**	127	53	38	42
	WHSC2-103 (A2, A33)	79	**168**	109	35	30	37
	HNRPL-140 (A2)	0	0	**184**	16	17	24
	SART3-109 (A33)	89	101	90	10	0	10
	WHSC2-141 (A2)	0	0	**96**	0	0	0
	UBE2V-43 (A2)	0	0	0	30	25	46
10 (A11, A33)	CypB-129 (A11, A33)	0	**22**	0	0	0	**4,109**
	Lck-449 (A11, A33)	17	0	**46**	0	0	0
	Lck-90 (A11, A33)	93	71	0	0	10	**17**
	SART3-734 (A11, A33)	53	43	53	64,000	52,180	6,140
	WHSC2-103 (A11, A33)	39	49	53	0	0	**20**
	SART3-109 (A11, A33)	40	40	0	0	0	**23**
Response rates (confidence intervals)		0.80 (0.2836–0.9949)			0.40 (0.0527–0.8534)		
Arm C (60 mg/1.5 ml)							
11 (A2, A26)	CypB-129 (A2)	0	0	0	0	0	**1,707**
	Lck-246 (A2)	0	**134**	**281**	0	0	0
	SART3-302 (A2)	0	**50**	0	6,110	5,797	**95,650**
	WHSC2-103 (A2)	45	0	0	0	0	**45**
	HNRPL-140 (A2)	49	0	0	0	0	**16**
	SART3-109 (A26)	0	0	0	0	0	0
	UBE2V-43 (A2)	0	0	**41**	14	10	**10,287**
	WHSC2-141 (A2)	60	0	0	0	0	0
12 (A24, A26)	EGFR-800 (A24)	0	0	0	0	0	**102**
	Lck-208 (A24)	0	0	0	11	10	**79**
	MRP3-1293 (A24)	0	0	0	0	0	**10**
	PSA-248 (A24)	0	0	0	331	7,724	**8,167**
	PTHrP-102 (A24)	0	0	0	38	34	37
	Lck-486 (A24)	0	0	0	11	12	**6,358**
	Lck-488 (A24)	0	**29**	0	17	15	**76**
	PSMA-624 (A24)	0	0	0	0	0	**72**
	PAP-213 (A24)	0	**146**	**36**	33	39	**152,340**
	SART3-109 (A24, A26)	0	0	0	0	0	**195**
13 (A24, A11)	CypB-129 (A11)	0	**25**	0	0	0	0
	EGFR-800 (A24)	0	0	0	0	0	0
	Lck-208 (A24)	0	0	0	0	0	0
	Lck-449 (A11)	16	**34**	0	0	0	0
	Lck-90 (A11)	17	**36**	20	23	26	17
	MRP3-1293 (A24)	0	0	0	0	0	0
	PSA-248 (A24)	0	0	0	0	0	0
	PTHrP-102 (A24)	0	0	0	0	0	0
	SART3-734 (A11)	24	**49**	31	108	116	147
	WHSC2-103 (A11)	0	**29**	**30**	85	159	**172**
	Lck-486 (A24)	0	0	0	0	0	0
	Lck-488 (A24)	0	0	0	14	15	0
	PSMA-624 (A24)	0	0	0	0	0	0
	PAP-213 (A24)	0	0	0	0	0	0

Table 4 continued

Patient no. (HLA)	HLA matching peptides (Target HLA)	CTL response (spots/10^5× cells)			IgG Response (FIU)		
		Pre	3rd	6th	Pre	3rd	6th
	SART3-109 (A24, A11)	35	0	0	0	0	0
14 (A2, A33)	CypB-129 (A2, A33)	0	0	0	0	0	**42**
	Lck-246 (A2)	0	**285**	**181**	0	10	0
	Lck-449 (A33)	0	**29**	**39**	17	18	19
	Lck-90 (A33)	44	28	24	45	63	**102**
	SART3-302 (A2)	0	0	0	20	20	21
	SART3-734 (A33)	77	28	24	13	13	10
	WHSC2-103 (A2, A33)	54	0	0	22	23	25
	HNRPL-140 (A2)	0	0	0	0	**10**	**11**
	SART3-109 (A33)	71	35	19	24	22	**86**
	WHSC2-141 (A2)	0	0	0	0	0	0
	UBE2V-43 (A2)	0	0	0	0	0	0
15 (A11)	CypB-129 (A11)	0	0	0	0	0	0
	Lck-449 (A11)	0	0	0	0	0	0
	Lck-90 (A11)	21	**56**	0	0	0	0
	SART3-734 (A11)	0	0	0	0	**77**	**136**
	WHSC2-103 (A11)	17	0	0	0	0	**23**
	SART3-109 (A11)	0	0	0	0	0	0
Response rates (confidence intervals)		0.80 (0.2836–0.9949)			1.00 (0.4782–1.0000)		
Total		0.60 (0.3229–0.8366)			0.73 (0.4490–0.9221)		

CTL cytotoxic T lymphocyte, *FIU* fluorescence intensity units, *HLA* human leukocyte antigen, *IgG* immunoglobulin G, *Pre* pretreatment

The values showing the increment are in bold

20 mg dose cohort. In addition, the frequency of MDSC in CTL-positive patients was lower than that in CTL-negative patients in the 20 mg dose cohort ($p = 0.019$, Wilcoxon signed rank test). The mean levels of plasma sCTLA-4 at pre-vaccination, and after the third and sixth vaccinations were 0.3, 0.2 and 0.3 ng/ml, respectively. These low levels of sCTLA-4 were similar when compared with those in healthy donors, as previously reported [22]. No difference in sCTLA-4 levels during vaccination was seen in any cohort.

Discussion

The toxicities related to the KRM-20 vaccine were limited to grade 1 or 2. The most common reported toxicity was grade 1 local injection site reactions, and a few cases experienced a grade 2 increase in liver enzymes or herpes zoster, in agreement with the results from our previously conducted personalized peptide vaccination [13, 18, 19].

The main objective of this phase I study was to determine the dose for subsequent efficacy trials through the study of immune dynamics, which was not well conducted in previous trials. Peptide vaccines with MART-1/Melan A or others were initially evaluated in a phase I setting, at doses ranging from 0.1 to 10 mg, and no toxicity was encountered even at the highest doses [23, 24]. A correlation between peptide dose and the generation of specific T cell responses from the PBMC of vaccinated patients was also not reported. Thus, neither safety nor efficacy can be assessed in patients with a blunted immune response. Our past study also reported that the maximum tolerated dose was not reached in a phase I study of personalized peptide vaccination, using 0.3–5 mg/peptide, and then 3 mg/peptide times 4 peptides (12 mg/body) was recommended for further clinical trials because of its tolerability and immune responses [18, 19]. Therefore, in the current study, we set 0.3 mg/peptide times 20 peptides (6 mg/body), 1 (20 mg) and 3 mg/peptide (60 mg). The 20 peptides were mixed with incomplete Freund's adjuvant (Montanide ISA-51VG) and prepared for emulsion at three different dose levels. The Montanide ISA51VG was used as an adjuvant in the current study, since it is the most popular in clinical use to induce cellular immunity and has been employed in the majority of peptide vaccine trials globally [25]. Because PBMCs contain low frequencies of antigen-specific T cells even after vaccinations, the current CTL response (ELIspot) assays have limited sensitivity to detect them directly ex vivo [26]. Therefore, in the current study, we stimulated and expanded antigen-specific T cells using in vitro cell culture with the specific antigens. Nevertheless, since in vitro-expanded cells do not necessarily give a better picture

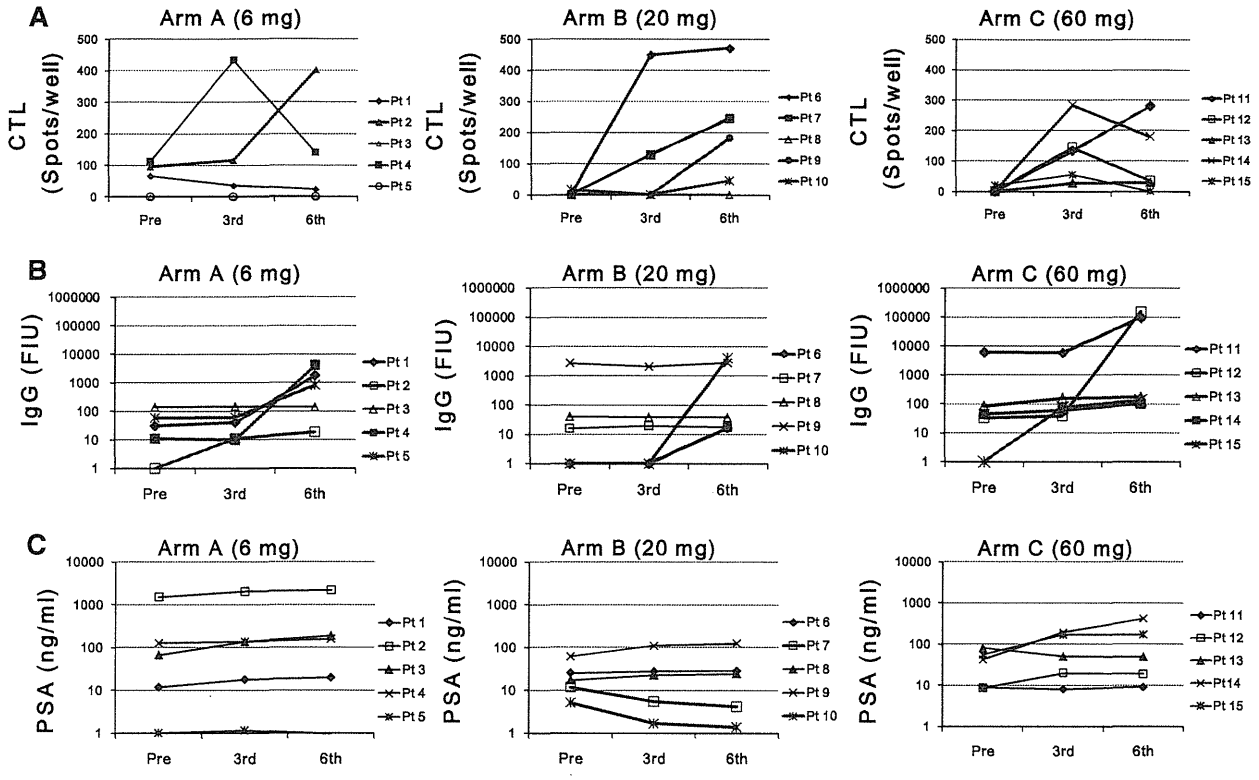

Fig. 1 Immune responses and PSA responses. HLA-matched best peptide-specific CTL (**a**) and IgG (**b**) responses, and PSA responses (**c**) in 15 patients divided by treatment dose (6, 20 or 60 mg) during the vaccination. Positive responses are highlighted in *blue*. CTL, cytotoxic T lymphocyte; FIU, fluorescence intensity units; IgG, immunoglobulin G; Pre, pretreatment; PSA, prostate-specific antigen

of the T cell activity present in vivo, the development of novel assays for detecting and monitoring antigen-specific T cells directly ex vivo is anticipated. In contrast, the multiplex bead-based Luminex technology allows high-throughput screening of IgG responses specific to large numbers of short peptides with high accuracy [20]. Our previous studies suggested the clinical significance of peptide-specific IgG responses as a surrogate biomarker in monitoring vaccine-induced immune responses [30]. Therefore, we measured the peptide-specific IgG responses as well as peptide-specific CTL responses for detecting and monitoring immune responses after vaccination. As a result, in all HLA-matched peptides, the CTL response after the third vaccination was boosted for 3 of 61, 12 of 45 and 11 of 50 peptides tested in patients receiving 6, 20 and 60 mg of KRM-20, respectively. That after the sixth vaccination was 4 of 61, 10 of 45 and 6 of 50 peptides, respectively, indicating that CTL response after the sixth vaccination largely decreased in the 60 mg cohort. From a view of both preexisting and boosted CTL activity, CTL activity was boosted in patients with 20 mg cohort, but not the other groups. These results indicated that CTL activity was augmented as early as after the third vaccination in patients with 20 and 60 mg cohorts followed by continued activation in 20 mg cohort and decline in 60 mg cohort. In addition, the MIED of KRM-20 in CTL or IgG response calculated by logistic regression model was 16 mg (0.8 mg/peptide) or 1.6 mg (0.3 mg/peptide), respectively. PSA responses were better in the 20 mg group with two PR and one NC. All these results suggest that 20 mg of KRM-20 could be recommended for a phase II study primarily because of lower levels of vaccine-induced suppression to CTL activity and PSA responses. However, the design to select the MIED of KRM-20 in this study has several limitations, including small numbers of patients in each arm and four different HLA types. Therefore, it might be difficult to compare the immune response rates with a wide confidence interval in different dose groups with small numbers of patients who have different HLA alleles. These issues shall be addressed in further studies with large numbers of patients are needed.

It has been well documented that efficiently primed T cells often lose their responsiveness to tumor antigens. This may be explained by a variety of mechanisms, including down-regulation or loss of tumor antigens, immunosuppression by T_{reg} and MDSC cells, and T cell inhibition mediated by checkpoint molecules, such as CTLA-4 and PD-1 [16, 26,

27]. In our exploratory analysis to evaluate effect of KRM-20 vaccine on such inhibitory mechanisms, we measured the frequency of T_{reg} and MDSC in PBMCs and sCTLA-4 in plasma, but no obvious differences were observed in the frequency of T_{reg} and MDSC and the level of sCTLA-4 during the vaccination. However, frequency of MDSC in CTL-positive patients was lower than that in CTL-negative patients in 20 mg dose cohort, suggesting that MDSC could be responsible for inhibition of CTL activity augmented by vaccination. Vaccination with 20 mg of KRM-20 might be better than that with the others from this point of view, although further studies with large numbers of patients are needed.

In this study, we used 20 mixed peptides containing several HLA alleles for patients with different HLA types. After the vaccination of 20 mixed peptides, HLA-matched peptides would be recognized by CTLs, but peptides with different HLA alleles would theoretically be metabolized without a biological effect. There might be concern about the competition between peptides for binding to the same HLA restriction element in KRM-20. Although peptide competition was not directly evaluated here, we detected CTL response to two or more peptides (range 2–6 peptides), restricted to HLA-A2, A24, A3 supertype or A26 alleles. This is consistent with what has been reported for other multiple peptide vaccines suggesting that competition for binding to the same HLA molecule is not significant enough to limit immunogenicity [28, 29]. Thus, multiple peptides can be combined and administered as the same mixture to elicit CD8+ T cell responses.

Although we have not directly tested whether the 20 peptides used for vaccination were naturally processed or not, we employed a cDNA expression technique to identify genes encoding 7 of the 12 antigens shown above, indicating that at least some of the peptides could be presented to the T cell receptor of CTL used as indicator cells for cloning. In addition, we confirmed that the CTL specific to the 20 peptides employed could recognize cancer cells expressing both corresponding antigens and HLA, but not those lacking either of them, suggesting that these peptide epitopes are processed and presented naturally by cancer cells. We also tested the reactivity of peptides to CTL and IgG from pre-vaccination samples of cancer patients using more than 1,000 peptide candidates derived from the 12 antigens shown above. Thereafter, 31 peptide candidates were selected for clinical trials of personalized peptide vaccination [5, 13]. The 20 peptides were further chosen among the 31 peptides based upon the higher reactivity in order to obtain pre-vaccination samples of CRPC patients. Therefore, the 20 peptides might be naturally recognized by the immune system of pre-vaccination CRPC patients through natural presentation to peptide-reactive T cells.

In summary, this phase I study of KRM-20 consisting of 20 mixed peptides derived from twelve TAA for patients with CRPC demonstrated the feasibility, safety, and rapid and high immune responses without changes of immunosuppressive cell subsets. Vaccination with 20 mg of KRM-20 could be recommended for further clinical trials based on tolerability and immune responses. With these encouraging results, a randomized, placebo-controlled, phase II study for CRPC is underway.

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Conflict of interest K Itoh is a consultant/advisory board member in Green Peptide Co. A.Yamada is a part-time executive of Green Peptide Co. No potential conflict of interest is disclosed by other authors.

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Personalized Peptide Vaccine for Treatment of Advanced Cancer

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Abstract: The field of cancer immunotherapy has moved forward drastically in the past 20 years, since many tumor-associated antigens (TAA) have been identified. Although various approaches for therapeutic cancer immunotherapies, including peptide-based vaccines, have been developed and clinically examined, the complexity and diversity of tumor cell characteristics and host immune cell repertoires seem to limit the therapeutic efficacy of this treatment modality. Considering the diversity of immune responses against heterogeneous tumor cells, tailored selections of vaccine antigens appropriate for individual patients could be a rational approach for developing effective cancer vaccines. We have developed a novel immunotherapeutic approach called personalized peptide vaccine (PPV), in which a maximum of four human leukocyte antigen (HLA)-matched vaccine peptides were selected based on the pre-existing host immunity before vaccination. We conducted a series of phase I and phase II clinical trials of PPV, which have shown better antigen-specific immune responses and promising clinical outcomes in patients with various types of advanced cancers. Further randomized phase III trials would be recommended to prove the clinical benefits of PPV. In addition, novel biomarkers for selecting patients who would benefit most from PPV remain to be identified.

Keywords: Advanced cancer, biomarker, cancer immunotherapy, clinical trial, peptide epitope, personalized peptide vaccine.

1. INTRODUCTION

The field of cancer immunology and immunotherapy has moved forward drastically in the past 20 years, since many different tumor-associated antigens (TAA) have been identified [1-5]. Various approaches for therapeutic cancer immunotherapies have been developed and clinically examined, including cancer vaccines using tumor cells, proteins, peptides, viral vectors, DNA, or dendritic cells, and great advances have been made in the clinical efficacy of cancer immunotherapy [1-5]. Notably, two novel immunotherapeutic agents have recently been approved by the US Food and Drug Administration (FDA) for patients with advanced cancer [6, 7]. In April 2010, sipuleucel-T (Provenge; Dendreon Corporation, Seattle, WA), an autologous antigen-presenting cell (APC) product designed to stimulate antigen-specific immune responses against human prostatic acid phosphatase (PAP), was approved for the first time by the US FDA for the treatment of patients with castration-resistant prostate cancer (CRPC). The FDA granted this approval after treatment with sipuleucel-T improved overall survival by 4.1 months [mean survival time (MST), 25.8 months vs 21.7 months] in the largest phase 3 randomized controlled trial (the IMPACT study) [6]. In addition, in March 2011 the FDA approved ipilimumab (Yervoy; Bristol-Meyers Squibb, Princeton, NJ), an immunomodulating antibody that blocks cytotoxic T-lymphocyte antigen 4 (CTLA-4), one of the immune checkpoint molecules in T cells, to treat advanced melanoma patients. In the phase III randomized controlled trial, this agent resulted in a 3-month improvement in overall survival with a disease control rate of 28.5%, where 60% of the responding patients maintained disease control for more than 2 years [7].

Moreover, there have been promising results in immunotherapeutic approaches to the treatment of various types of advanced cancers, although they have not yet been officially approved. For example, blocking antibodies against a T-cell co-inhibitory receptor, programmed death 1 (PD-1), and one of its ligands, PD-ligand 1 (PD-L1), which have been reported to contribute to tumor cell escape from host immune surveillance, have shown feasible results against various types of cancers [8, 9]. Topalian *et al.* demonstrated that anti-PD-1 antibody revealed objective responses in approximately 20 to 25% of patients with non-small-cell lung cancer (NSCLC), melanoma, or renal-cell cancer [8]. Brahmer *et al.* reported that anti-PD-L1 antibody, which blocks the interaction between PD-1 and PD-L1, could induce durable tumor regression (objective response rates of 6% to 17%) and prolonged stabilization of disease (12% to 41% of patients at 24 weeks) in patients with advanced cancers, including NSCLC, melanoma, and renal-cell cancer [9]. Currently, these promising advancements are generating great optimism and heightened enthusiasm for the further development of cancer immunotherapies.

In addition to these significant advances, many other clinical trials of cancer immunotherapies have been under way to show beneficial therapeutic effects in patients compared to existing treatments [1-5]. In this review, we discuss the recent advances in peptide-based cancer vaccines. In par-

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ticular, we describe the details of our novel immunotherapeutic approach, called the personalized peptide vaccine (PPV), which has demonstrated promising results for advanced cancer patients in a series of clinical trials.

2. PERSONALIZED PEPTIDE VACCINE (PPV)

In 1991, Boon *et al.* for the first time reported a cDNA-expression cloning technique to identify TAA [10]. Subsequently, serologic analysis of recombinant cDNA expression libraries (SEREX), another technique for detecting TAA using autologous antibodies, was introduced for the identification of genes recognized by the host immune system [11]. Such advancement of molecular biological and immunological techniques has helped identify a large number of TAA and peptide epitopes applicable as cancer vaccines [12-14]. Since 1995, when Hu *et al.* reported the first clinical trial of the vaccination of a peptide derived from melanoma antigen gene-1 (MAGE-1) [15], many clinical trials of peptide vaccines have been reported [16, 17]. In earlier stages of clinical trials of peptide vaccines, one to several human leukocyte antigen (HLA) class I-restricted peptides emulsified with Montanide ISA51, a clinical grade of Freund's incomplete adjuvant, were employed. Although the early phase clinical trials demonstrated the feasibility and good toxicity profile of this approach, most of the late-phase randomized trials, other than few exceptions [18], failed to show beneficial therapeutic effects in patients compared to existing treatments [16, 17]. Therefore, a variety of new types of peptide-based vaccines have been developed [19, 20] (Fig. **1**). We first discuss our novel peptide-based approach, PPV, in which multiple vaccine antigens appropriate for each patient are selected from a panel of vaccine candidates based on pre-existing host immunity.

2.1. Rationale for Personalized Selections of Vaccine Peptides

Cancer patients possess anti-tumor immunity, which may depend strongly on both the tumor cell characteristics and the immunological status of the host [21-24]. The anti-tumor immunity might differ widely among individuals, since the tumor cell characteristics and the host immune cell repertoires are quite diverse and heterogeneous among patients, even among those with identical HLA types and the same pathological types of cancer. Nevertheless, before patients are enrolled in clinical trials of cancer vaccines, the expressions of vaccine antigens in tumor cells are sometimes confirmed, but the immunological statuses of the hosts are rarely evaluated. Considering the complexity and diversity of the host immune cell repertoires, it is likely that vaccine antigens that are selected and administered without considering the host immunological status might not efficiently induce beneficial anti-tumor immune responses [24]. Since, in most clinical trials of therapeutic cancer vaccines, common antigens are employed for vaccination independently of the immunological status of patients [16, 17], the low clinical efficacies might be explained at least in part by mismatches between the vaccine antigens and the host immune cell repertoires.

To evaluate the host immune cell repertoires, we examine patients' pre-existing immunity to a panel of vaccine candidates before vaccination and select appropriate vaccine antigens with immunological memory in each patient [25]. Vaccine antigens, to which patients already possess antigen-specific immunological memory, are expected to cause quick and strong secondary immune responses after vaccination (Fig. **2**). In contrast, vaccinations with inadequate antigens without immunological memory could not easily provide

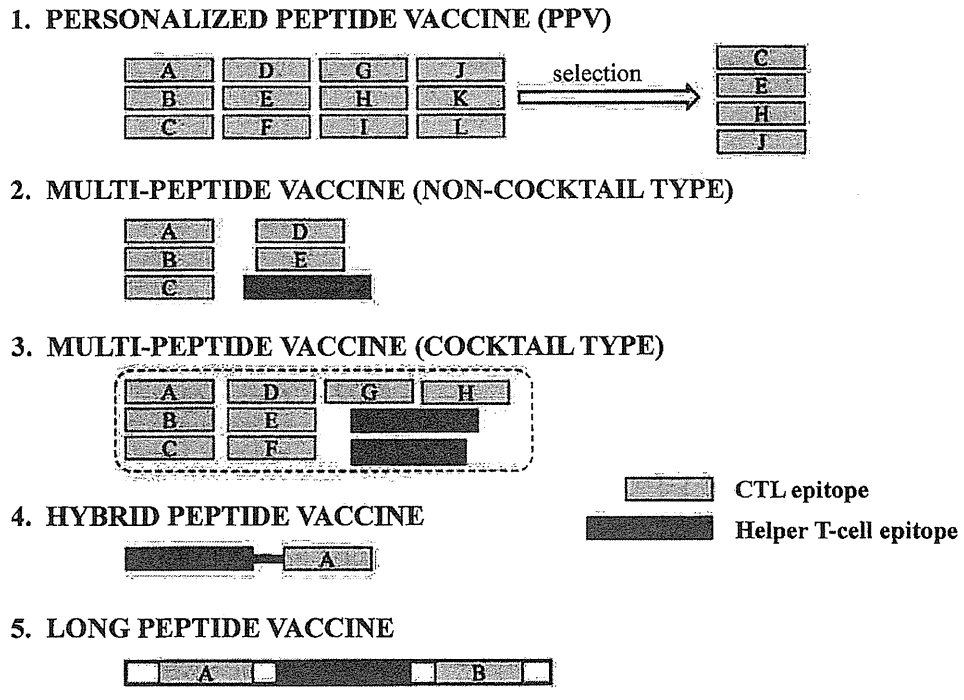

Fig. (1). Recent development of new types of peptide-based vaccines. Examples of new types of peptide-based vaccines are shown. Gray and black boxes indicate CTL and helper T-cell epitopes, respectively.

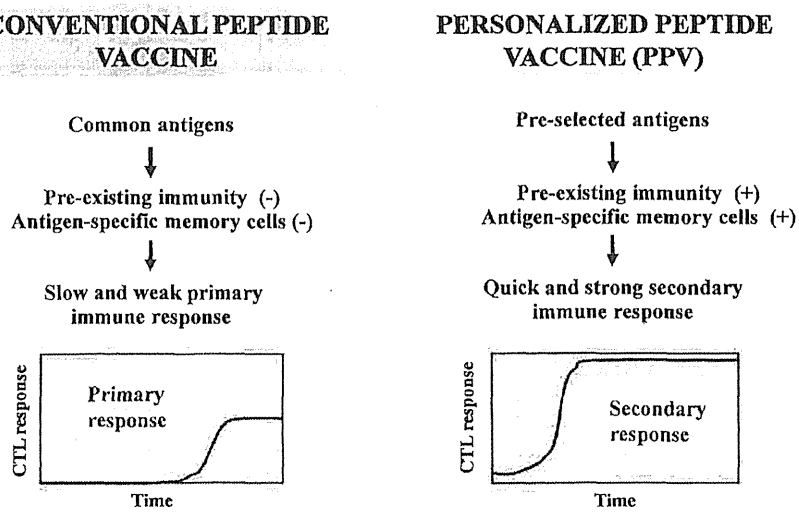

Fig. (2). Rationale of personalized peptide vaccine. In conventional peptide vaccines without pre-existing immunity, patients without immunological memory to vaccine antigens would take more time to develop effective anti-tumor immune responses because several rounds of repeated vaccinations might be required to prime antigen-specific naive T cells to functional effector cells. In personalized peptide vaccines with the pre-existing immunity, patients with antigen-specific immunological memory are expected to show quick and strong secondary immune responses to them.

clinical benefits, especially in advanced cancer patients who show rapid disease progression [26]. In light of this, it would be quite reasonable to select vaccine antigens on the basis of the pre-existing immune cell repertoires in each patient.

Cancer cells can develop various mechanisms to accelerate malignant behavior [21]. For example, it has been well recognized that cancer cells might escape the host's immunological surveillance. After the interaction/competition between tumor cells and host immune cells, tumor cell variants resistant to the immunological pressure often emerge through the selection of mutants with reduced antigenicity [21]. Therefore, the selection and administration of multiple vaccine antigens could reduce the risk of tumor escape through the existence and/or induction of antigen-negative variants escaping antigen-specific immune responses [22, 27], since it would be rare for tumor cells to simultaneously lose all of the multiple antigens selected for vaccination.

Collectively, our new concept of "personalized" cancer vaccine formulation, where multiple peptide antigens are selected for vaccination by the pre-existing host immunity from a list of vaccine candidates, may confer several advantages, including the possibility of bypassing both immunological diversity and tumor heterogeneity.

2.2. PPV Procedures

For PPV, a maximum of four peptides are selected based on the results of HLA typing and the pre-existing immune responses specific to each of the 31 HLA class I-restricted cytotoxic T lymphocyte (CTL) epitope peptides with minimal optimal lengths (9-mer or 10-mer): 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 (Table 1). These peptides were identified mainly through the cDNA expression cloning method with tumor-infiltrating T-lymphocyte lines [25, 28-34]. The safety and potential immunological effects of these vaccine candidates have been demonstrated in clinical studies [25, 35, 36]. It should be noted that we currently employ these 31 CTL epitopes, which are also shown to induce antigen-specific B-cell immune responses, as vaccine antigen candidates for PPV, since it has been suggested that a CTL peptide with the ability to induce antigen-specific B-cell responses could provide more effective immune responses than a CTL peptide without it [37, 38].

Although short peptide epitopes with minimal optimal lengths have been reported to bear the potential to induce immune tolerance rather than activate antigen-specific immune responses [39-41], our PPV formulation with short epitopes has been demonstrated to efficiently induce antigen-specific IFN-γ-producing $CD8^+$ T cells, but not tolerance to them, possibly because only immunogenic epitopes are selected in each patient by screening before vaccination. Although long synthetic peptides have shown excellent immune responses and promising clinical results in some clinical trials [42, 43], we do not currently use long peptides for PPV, since they may contain undesirable T-cell epitopes that activate other immune cells, such as T helper 2 cells and/or regulatory T cells [44, 45], which could negatively affect beneficial antigen-specific immune responses.

Different peptides have their own different binding affinities to the corresponding HLA molecules. Therefore, if multiple CTL-epitope peptides with different HLA-binding affinities are loaded to APCs, the individual peptides may compete with each other to bind HLA molecules on the APCs [46]. For PPV, to prevent such competition among peptides at the vaccinated sites, a maximum of 4 immunogenic peptides selected from the 31 different vaccine candidates are individually mixed with incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France) and subcutaneously injected at different sites, but not at a single site as a mixture. Regarding the vaccination schedule,

Table 1. Peptide candidates used for personalized peptide vaccine (PPV).

Peptide Name	HLA Restriction	Original Protein	Position	Amino Acid Sequence
CypB-129	A2 A3sup	Cyclophilin B	129-138	KLKHYGPGWV
EGFR-800	A24	EGF-R	800-809	DYVREHKDNI
EZH2-735	A24	EZH2	735-743	KYVGIEREM
HNRPL-140	A2	HNRPL	140-148	ALVEFEDVL
HNRPL-501	A2 A26	HNRPL	501-510	NVLHFFNAPL
Lck-90	A3sup	p56 lck	90-99	ILEQSGEWWK
Lck-208	A24	p56 lck	208-216	HYTNASDGL
Lck-246	A2	p56 lck	246-254	KLVERLGAA
Lck-422	A2 A3sup	p56 lck	422-430	DVWSFGILL
Lck-449	A3sup	p56 lck	449-458	VIQNLERGYR
Lck-486	A24	p56 lck	486-494	TFDYLRSVL
Lck-488	A24	p56 lck	488-497	DYLRSVLEDF
MAP-432	A2 A26	ppMAPkkk	432-440	DLLSHAFFA
MRP3-503	A24	MRP3	503-511	LYAWEPSFL
MRP3-1293	A24	MRP3	1293-1302	NYSVRYRPGL
PAP-213	A24	PAP	213-221	LYCESVHNF
PAP-248	A3sup	PAP	248-257	GIHKQKEKSR
PSA-248	A24	PSA	248-257	HYRKWIKDTI
PSMA-624	A24	PSMA	624-632	TYSVSFDSL
PTHrP-102	A24	PTHrP	102-111	RYLTQETNKV
SART2-93	A24	SART2	93-101	DYSARWNEI
SART2-161	A24	SART2	161-169	AYDFLYNYL
SART3-109	A24 A3sup A26	SART3	109-118	VYDYNCHVDL
SART3-302	A2	SART3	302-310	LLQAEAPRL
SART3-309	A2	SART3	309-317	RLAEYQAYI
SART3-511	A3sup	SART3	511-519	WLEYYNLER
SART3-734	A3sup	SART3	734-742	QIRPIFSNR
UBE-43	A2	UBE2V	43-51	RLQEWCSVI
UBE-85	A2	UBE2V	85-93	LIADFLSGL
WHSC2-103	A2 A3sup A26	WHSC2	103-111	ASLDSDPWV
WHSC2-141	A2	WHSC2	141-149	ILGELREKV

A3sup: HLA-A3 supertype (A3, A11, A31, or A33); EGF-R: Epidermal Growth Factor Receptor; EZH2: enhancer of zeste homolog 2; HNRPL: heterogeneous nuclear ribonucleoprotein L; ppMAPkkk: partial putative mitogen-activated protein kinase kinase kinase; MRP3: multidrug resistance-associated protein 3; PAP: Prostatic acid phosphatase; PSA: prostate specific antigen; PSMA: Prostate specific membrane antigen; PTHrP : parathyroid hormone-related peptide; SART2: squamous cell carcinoma antigen recognized by T cells 2; SART3: squamous cell carcinoma antigen recognized by T cells 3; UBE2V: ubiquitin-conjugated enzyme variant Kua; WHSC2: Wolf-Hirschhorn syndrome candidate 2.

the selected peptides are administered weekly for at least the first cycle of six vaccinations, since a clear trend toward better immune responses was observed among the patients who underwent the weekly administration protocol compared to those who underwent a bi-weekly protocol in our previous clinical trials [47].

One of the noticeable characteristics of our PPV formulation is that it screens vaccine antigen candidates before vaccination, based on CTL-precursor frequencies and/or immunoglobulin G (IgG) titers specific to each of the candidates in pre-vaccination blood samples from each patient [25]. In the earlier stage of translational studies of PPV, pre-existing immunity was defined by the frequencies of CTL precursors in pre-vaccination peripheral blood mononuclear cells (PBMC) by detecting peptide-specific IFN-γ production by enzyme-linked immunosorbent assay (ELISA) [47-51]. However, we are currently evaluating the pre-existing im-

munity to vaccine candidates by measuring peptide-specific IgG titers in pre-vaccination plasma by the multiplex bead-based Luminex assay rather than CTL precursor frequencies, since the performance characteristics, such as the sensitivity and reproducibility, of the current T-cell assays are sometimes unsatisfactory for detecting low frequencies of antigen-specific CTL [52, 53]. In contrast to the drawbacks inherent to T-cell assays, the multiplex bead-based Luminex technology that we have developed to monitor B-cell responses allows simple, quick, and highly reproducible high-throughput screening and monitoring of IgG responses specific to a large number of peptide antigens with a tiny amount of plasma [36, 54, 55]. Indeed, the selection of vaccine antigens based on IgG titers seemed to be useful for predicting CTL boosting after vaccination in our clinical trials. The predictive power of evaluating the existence of antigen-specific CTL precursors solely by the humoral responses before vaccination could be estimated at around 50% when four peptides were chosen for PPV in each patient [56, 57].

2.3. Clinical Trials of PPV for Advanced Cancers

A series of phase I, I/II, and II clinical trials of PPV has been conducted in the past several years for various types of advanced cancer patients. Table **2** summarizes the immune and clinical responses of advanced cancer patients treated with PPV. In the following sections, we provide some detailed information on these clinical studies.

2.3.1. Castration-Resistant Prostate Cancer (CRPC)

In phase I studies of PPV for advanced HLA-$A2^+$ or HLA-$A24^+$ CRPC, we have reported increased cellular and humoral immune responses and decreased PSA levels in some patients [58, 59]. In a phase I dose-escalation study of PPV (1, 3, and 5 mg/peptide injection) for HLA-$A24^+$ CRPC, we have also demonstrated that a dose of 3 mg/peptide injection showed better cellular immune responses to vaccine peptides than either 1 or 5 mg/peptide injections, although the maximum tolerated dose (MTD) was not determined [56]. In addition, in a phase I/II study of 58 HLA-$A2^+$ or HLA-$A24^+$ CRPC patients, a combination of PPV and low-dose estramustine phosphate (EMP) showed a median survival time (MST) of 17 months (95% confidence interval (95% CI), 12 to 25 months), along with a decreased serum PSA level in the majority (76%) of patients [60]. The same study also revealed that fewer lymphocytes, negative immunological responses to vaccine antigens, and poor performance status were independent predictors of disease-related death [60].

Subsequently, we conducted a randomized phase II trial to compare PPV plus low-dose EMP with standard-dose EMP in HLA-$A2^+$ or HLA-$A24^+$ CRPC patients. The patients receiving PPV in combination with low-dose EMP showed a significantly longer progression-free survival [MST, 8.5 months vs 2.8 months; hazard ratio (HR), 0.28 (95% CI, 0.14-0.61); $P = 0.0012$] and overall survival [MST, undefined vs 16.1 months; HR, 0.30 (95% CI, 0.1-0.91); $P = 0.0328$] than those receiving standard-dose EMP alone, suggesting the efficacy of this combination therapy [61]. In another phase II study, we compared docetaxel-based chemotherapy (DBC)-resistant CRPC patients undergoing PPV (n =20) with a historical control (n = 17). MSTs from the failure of previous DBC treatments were 17.8 and 10.5 months in patients treated with and without PPV, respectively [62]. These promising results suggested that PPV warrants further study as a novel therapy for CRPC patients, even for those with progressive disease following DBC treatment. A phase III randomized clinical trial of PPV is currently under way in DBC-resistant CRPC patients.

2.3.2. Malignant Glioma

In a phase I clinical study, we demonstrated the feasibility of PPV for HLA-$A2^+$ or HLA-$A24^+$ advanced malignant glioma patients [47]. The clinical responses of 27 patients who received more than six vaccinations were partial response (PR) in 5, stable disease (SD) in 8, and progressive disease (PD) in 8 patients, with a MST of 20.7 months. Significant levels of IgG specific to vaccine peptides were detected after vaccination in the tumor cavity or spinal fluid obtained from patients who had shown favorable clinical responses. Another phase I clinical trial in HLA-$A24^+$ patients with recurrent or progressive GBM also showed the safety and increased immune boosting of PPV with potential clinical benefits, with a MST of 10.6 months even after failure of the standard temozolomide treatment [57]. On the basis of these promising results, double-blind randomized phase III trials are under way in GBM patients resistant to the standard treatment.

2.3.3. Pancreatic Cancer and Biliary Tract Cancer

We have conducted a phase I trial of PPV in 13 HLA-$A2^+$ or HLA-$A24^+$ patients with advanced pancreatic cancer, where the patients were treated by PPV at three different doses (1, 2, or 3 mg/peptide) in combination with gemcitabin (GEM) [63]. This combination therapy was well tolerated, and 11 of 13 patients (85%) showed reduced tumor sizes and/or levels of tumor markers. Peptide-specific CTL responses were augmented at each dose level, and the increment of peptide-specific IgG antibodies was dependent on the peptide dose. These findings suggested that GEM did not inhibit the immune responses induced by PPV. Subsequently, we conducted a phase II trial of PPV in combination with GEM to evaluate the safety, clinical efficacy, and antigen-specific immune responses as a front-line therapy for 21 HLA-$A2^+$ or HLA-$A24^+$ nonresectable patients with advanced pancreatic cancer [64]. This combination therapy was also well tolerated, and the best clinical responses were PR in 7, SD in 9, and PD in 5 patients. The MST of all 21 patients was 9 months with a 1-year survival rate of 38%, which was better than that reported for GEM alone (MST of 5.7 months with a 1-year survival rate of 18%) [65]. Importantly, the MST was 15 months in patients who showed immunological responses to vaccine peptides.

We also conducted a phase II clinical trial of PPV in 25 HLA-$A2^+$ or HLA-$A24^+$ chemotherapy-resistant patients with advanced biliary tract cancer [66]. When two to four vaccine peptides selected by pre-existing immunity were administered to the patients in this study, humoral and/or T-cell responses specific to the vaccine antigens were substantially induced in a subset of the patients without severe adverse events. Greater numbers of selected and vaccinated peptides were significantly favorable factors for overall survival (HR = 0.258, 95% CI = 0.098-0.682, $P = 0.006$) in this study (Table **3**).

Table 2. List of clinical trials of personalized peptide vaccines (PPV) for advanced cancer.

Organ	Disease condition	Phase of trial	HLA restriction	Combined treatment	No. of Patients	Clinical response	MST (months)	Toxicities (Grade 3/4)	Humoral response (%)	Cellular response (%)	Refer-ence
Prostate (CRPC)	Advanced	I	A24	-	10	SD 50%	NA	-	60	40	[58]
Prostate (CRPC)	Advanced	I	A24	EMP	13	PR 63%	24	G3, 5%	91	55	[114]
Prostate (CRPC)	Advanced	I	A2	-	10	SD 30%	22	-	70	40	[59]
Prostate (CRPC)	Advanced	I/II	A24	EMP	16	PR 43%	17	-	50	71	[115]
Prostate (CRPC)	Advanced	I/II	A2/A24	EMP	58	PR 24%	17	G3, 7%	88	78	[60]
Prostate (CRPC)	Advanced	I	A24	EMP	15	PR 13%	24	-	47	67	[56]
Prostate (CRPC)	Advanced	II (Ran-domized)	A2/A24	EMP	57	8.5M vs 2.8M (PFS)	22.4M vs 16.1M	-	64	50	[61]
Prostate (CRPC)	Advanced	II	A2/A24/ A3sup/A26	-	42	PR 12%	17.8	-	44	34	[62]
Prostate	Localized	II	A24	-	10	PR 20%	NA	-	80	80	[116]
Brain	Advanced malignant glioma	I	A2/A24	-	21	PR 24%, SD 38%	NA	-	40 - 64	50 - 82	[47]
Brain	Advanced GBM	I	A24	-	12	PR 17%, SD 42%	10.6	-	17	75	[57]
Pancreas	Advanced	I	A2/A24	GEM	13	PR 15%, SD 54%	7.6	-	69	69	[63]
Pancreas	Advanced	II	A2/A24	GEM	21	PR 33%, SD 43%	9	-	72	78	[64]
Biliary tract	Advanced	II	A2/A24/ A3sup/A26	Chemo-therapy	25	SD 32%	NA	G3, 4%	35	47	[66]
Stomach	Advanced	I	A2/A24	-	13	SD 45%	NA	-	80	50	[67]
Stomach Colorectal	Advanced	I/II	A2/A24	S-1	11	SD 36%	NA	G3, 18%	81	63	[69]
Colorectal	Advanced	I	A24	-	10	PR 10%	NA	-	70	50	[68]
Colorectal	Metastatic	I	A2/A24	UFT UZEL	13	SD 43%	19.6	G3, 7.7%	69	85	[70]
Lung	Advanced	I	A24	-	10	SD 80%	15.2	-	40	40	[50]
Lung	Advanced (NSCLC)	II	A2/A24 A3sup/A26	Chemo-therapy	41	SD 56%	10.1	G3, 7%	49	34	[71]
Lung	Advanced (SCLC)	II	A2/A24 A3sup/A26	Chemo-therapy	10	SD 20%	6.2	G3, 4%	83	83	[72]

(Table 2) contd....

Organ	Disease condition	Phase of trial	HLA restriction	Combined treatment	No. of Patients	Clinical response	MST (months)	Toxicities (Grade 3/4)	Humoral response (%)	Cellular response (%)	Reference
Urothelial	Advanced	I	A2/A24	-	10	CR 10%, PR 10%	24	-	80	80	[73]
Kidney	Metastatic	I	A2/A24	-	10	SD 60%	23	-	80	5	[74]
Uterine, Ovary	Recurrent	I	A2/A24	-	14	SD 36%	NA	G3, 8%	86	86	[49]
Skin	Malignant melanoma	I	A2/A24	-	7	SD 43%	NA	-	57	86	[51]

CRPC: castration-resistant prostate cancer; GBM: glioblastoma multiforme; SCLC: small cell lung cancer; NSCLC: non-small cell lung cancer; A3sup: HLA-A3 supertype (A3, A11, A31, or A33); EMP: estramustine phosphate; GEM: gemcitabine; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; PFS: progression-free survival; MST: median survival time; NA: not assessed; M: months.

Table 3. Biomarkers for personalized peptide vaccines (PPV) for advanced cancer.

Type of cancer	Factor	Statistical analysis (HR, 95% CI, P value)	Reference
Miscellaneous (n = 500)[a]	Performance status (1, 2, 3 vs 0)	HR = 2.295; 95% CI, 1.653 - 3.188; P < 0.0001	[36]
	Lymphocyte counts (<1500μL vs > 1500μL)	HR = 1.472; 95% CI, 1.099 - 1.972, P = 0.0095	
	IgG responses to antigens after vaccination (no vs yes)	HR = 1.455; 95% CI, 1.087 - 1.948, P = 0.0116	
Prostate (CRPC, n = 40)	IL-6	(Not determined)	[81]
	MDSC	(Not determined)	
Non-small lung cell cancer (n =41) [a]	C-reactive protein (CRP)	HR = 10.115, 95% CI = 2.447 – 41.806, P = 0.001	[71]
Biliary tract (n = 25)[a]	IL-6	HR = 1.123, 95% CI = 1.008 -1.252, P = 0.035	[66]
	Albumin	HR = 0.158; 95% CI, 0.029 - 0.860; *P* = 0.033	
	Numbers of vaccine peptides	HR = 0.258, 95% CI = 0.098-0.682, *P* = 0.006	

[a]Potential biomarkers for PPV were determined by multivariate Cox regression analyses.; HR: hazard ratio; CI: confidence interval; CRPC: castration-resistant prostate cancer; MDSC: myeloid-derived suppressor cells.

2.3.4. Gastric Cancer and Colorectal Cancer

In a phase I clinical trial of PPV in 13 HLA-$A2^+$ or HLA-$A24^+$ patients with advanced gastric cancer (9 nonscirrhous and 4 scirrhous), prolonged survival was observed in patients who showed cellular and humoral immune responses to the vaccine peptides in the post-vaccination blood samples, including all 4 patients with the scirrhous type [67]. In addition, a phase I clinical trial of PPV in 10 HLA-$A24^+$ patients with advanced colorectal cancer showed one PR and one SD, each continuing for more than 6 months [68].

In a phase I/II clinical trial of PPV in combination with three different doses (20, 40, or 80 mg/m^2/day) of oral administration of a 5-fluorouracil derivative, S-1, for 11 HLA-$A2^+$ or HLA-$A24^+$ advanced gastric or colorectal cancer patients [69], the combined administration of the standard dose (80 mg/m^2/day) of S-1 did not inhibit immunological responses to vaccine antigens, but instead maintained or augmented them. In another phase I clinical trial for 13 HLA-$A2^+$ or HLA-$A24^+$ metastatic colorectal cancer patients [70], the combined treatment of PPV and the oral administration of a 5-fluorouracil derivative, UFT, and calcium folinate, UZEL, proved to be safe and to induce good antigen-specific immune responses. In this trial, IgG responses to the vaccine peptides correlated well with overall survival. These encouraging results suggest that combined treatment with PPV and standard chemotherapeutic agents might be promising for advanced gastric and colorectal cancers.

2.3.5. Lung Cancer

The prognosis of advanced lung cancer patients remains very poor, with a MST of around 6-10 months. Phase I and II studies of PPV in a small number of patients with refractory NSCLC demonstrated that PPV was safe and well tolerated,