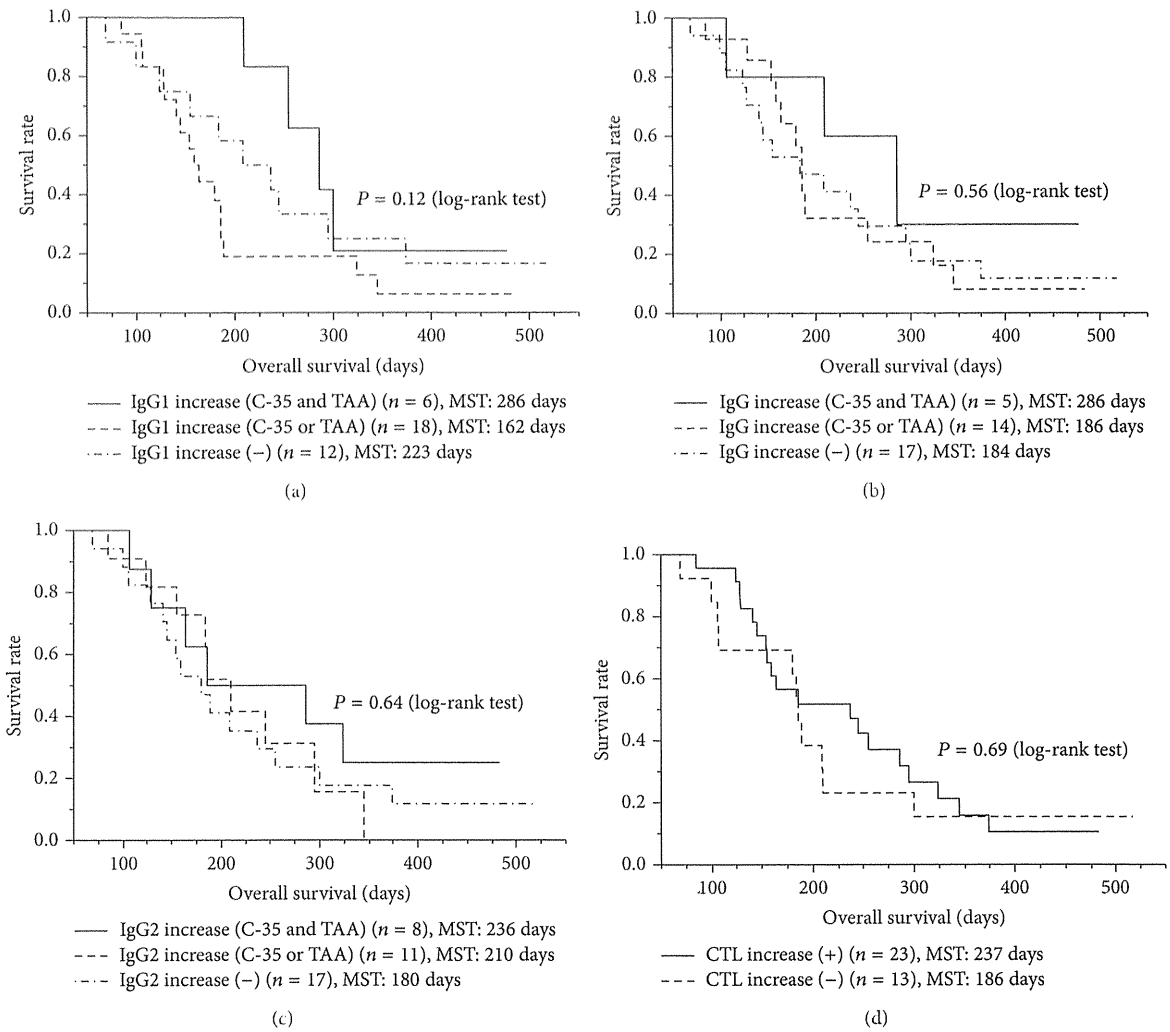

FIGURE 2: Immune response and overall survival. Association between immune responses and OS was examined with the Kaplan-Meier method, and a comparison of the survival curves was performed with the log-rank test. (a) The peptide-specific IgG1 response was potentially prognostic ($P = 0.12$ by log-rank test, $P = 0.06$ by Wilcoxon test); all 6 patients showing increased IgG1 responses to both the C-35 peptide and TAA-derived peptides survived more than 210 days, and their MST (286 days) tended to be longer than that of patients showing increased IgG1 responses to either peptide (162 days) or that of patients showing no increase to any peptide (223 days). (b) The peptide-specific IgG response was not prognostic ($P = 0.56$). (c) The peptide-specific IgG2 response was not prognostic ($P = 0.64$). (d) The peptide-specific CTL response was not prognostic ($P = 0.69$).

with sorafenib were 186 or 174 days, respectively. No grade 4 or 5 adverse events were observed in patients with PPV alone, whereas a grade 5 adverse event (pleural infection) was observed in a patient receiving PPV and sorafenib. These results suggested that the combination of sorafenib and PPV had no additive benefit, although the scale of the study was small.

From the viewpoint of biomarkers, the peptide-specific IgG1 response was suggested to be a potentially prognostic factor in this study, since all 6 patients showing boosted IgG1 responses to both C-35 peptide and TAA-derived peptides survived more than 210 days, and their MST (286 days) tended to be longer than that of patients showing boosted IgG1 responses to either peptide alone (162 days) or that of patients showing no increase in response to any peptide (223 days) ($P = 0.12$ by log-rank test, $P = 0.06$ by Wilcoxon test). In contrast, the peptide-specific IgG2 response did not show prognostic significance. Since IgG1, but not IgG2, is known to enhance antibody-mediated opsonization and phagocytosis of antigens, peptide-specific IgG1 may enhance antitumor immunity through phagocytosis and cross-presentation of antigen peptides [23]. Further studies will be needed to clarify the mechanisms.

In contrast to IgG1 responses as a potential prognostic biomarker, the peptide-specific CTL response was not well correlated with OS in these patients under PPV. This may

have been mainly due to the small size of patient numbers. Indeed, we suggested that the peptide-specific IgG response was more useful than the peptide-specific CTL response as a prognostic biomarker for patients under PPV, primarily because monitoring of IgG responses shows higher sensitivity than that of CTL responses [24].

5. Conclusion

The current study indicated that PPV with both a HCV-derived CTL epitope peptide and 31 peptides from TAAs could be recommended for the next step of a clinical trial in HCV-positive advanced HCC patients, because of safety and strong immune induction.

Conflict of Interests

One of the authors, Akira Yamada, is a board member of the Green Peptide Company, Ltd. Both Kyogo Itoh and Akira Yamada own stock in the Green Peptide Company, Ltd. Kyogo Itoh received research funds from Taiho Pharmaceutical Company, Ltd. The other authors declare that they have no competing interests.

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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

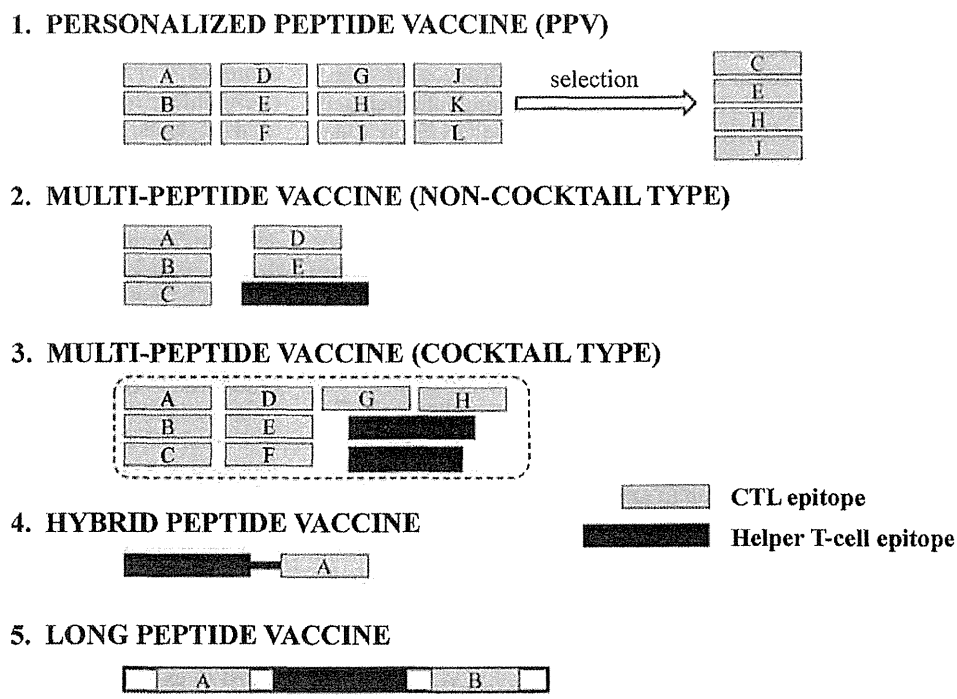

Fig. (1). Recent development of new types of peptide-based vaccines. Examples of new types of peptide-based vaccines are shown. Gray and black boxes indicate CTL and helper T-cell epitopes, respectively.

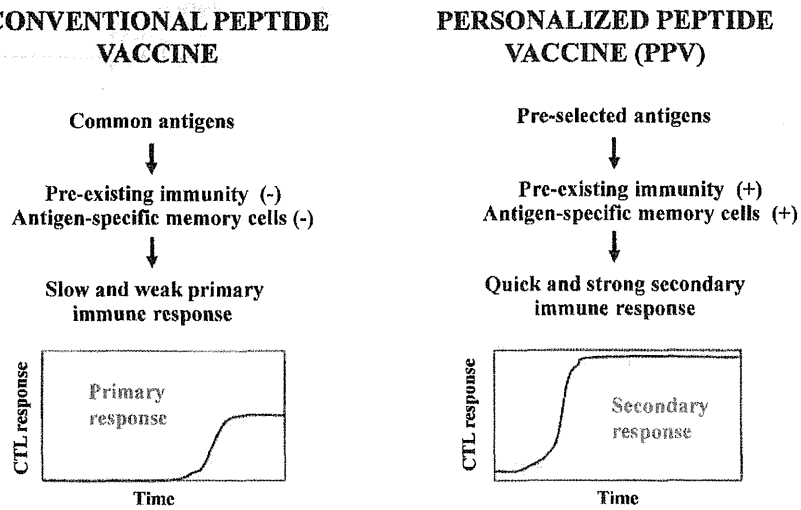

Fig. (2). Rationale of personalized peptide vaccine. In conventional peptide vaccines without pre-existing immunity, patients without immunological memory to vaccine antigens would take more time to develop effective anti-tumor immune responses because several rounds of repeated vaccinations might be 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,

with no major adverse effects, and that PPV treatment resulted in longer survival (MST of 10.1 or 15.2 months) [50, 71]. A clinical study in 10 advanced small cell lung cancer (SCLC) also showed the safety and feasibility of PPV [72].

2.3.6. Urothelial Cancer

A phase I clinical trial of PPV was conducted in 10 HLA-$A2^+$ or HLA-$A24^+$ refractory urothelial cancer patients [73]. In this study, some patients treated by PPV showed clear clinical responses as evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST) criteria with boosted immune responses: CR in 1, PR in 1, and SD in 2 patients. These 4 responders showed better progression-free survival (MST, 21 months) and overall survival (MST, 24 months), suggesting the potential clinical efficacy of PPV for advanced urothelial cancer.

2.3.7. Other Cancers

We also conducted phase I clinical trials for other advanced cancers, including metastatic renal cell carcinoma (RCC) [74], gynecologic cancers [49], and malignant melanoma [51]. All of these studies demonstrated that PPV was safe and well tolerated with no major adverse effects, and that good immune responses to vaccine antigens were induced in many of the patients after PPV. Further clinical trials would be required to clearly prove the clinical benefits of PPV in these cancers.

2.4. Biomarkers for PPV (Table 3)

Recent clinical trials of cancer immunotherapies, including peptide-based cancer vaccines, have demonstrated that only a subset of patients show clinical benefits. Furthermore, unexpectedly, some large clinical trials in the past several years have demonstrated that cancer vaccines might sometimes show worse clinical outcomes [75, 76]. It would thus be important to identify predictive biomarkers that could accurately assess anti-tumor immune responses and predict patient prognosis following the administration of cancer vaccines. In some clinical trials, several post-vaccination biomarkers, including CTL responses, Th1 responses, delayed-type hypersensitivity (DTH), and autoimmunity, have been reported to be associated with clinical responses.[77-80]. However, there are currently no validated biomarkers for cancer vaccines in widespread use.

To identify biomarkers for PPV, we statistically reviewed 500 advanced cancer patients undergoing PPV from October 2000 to October 2008 [36]. Both lymphocyte counts before vaccination (P = 0.0095) and increased IgG response (P = 0.0116) to the vaccine peptides after vaccination, along with performance status (P < 0.0001), were well correlated with overall survival. In CRPC patients treated with PPV (n = 40), a comprehensive study of soluble factors assessed by multiplexed bead array in plasma and gene expression profiles by DNA microarray in PBMC demonstrated that higher IL-6 level and granulocytic myeloid-derived suppressor cells (MDSC) in the peripheral blood before vaccination were closely related to poorer prognosis in the vaccinated patients [81]. By multivariate Cox regression analyses in patients with refractory NSCLC (n = 41), 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) [71]. In addition, in refractory biliary tract cancer patients (n = 25), multivariate Cox regression analyses showed that 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] [66].

Collectively, these findings suggested that less inflammation may contribute to better responses to PPV, indicating that the evaluation of inflammatory factors before vaccination could be useful for selecting cancer patients who are appropriate for PPV (Table 3). An early phase clinical trial is under way to reveal whether or not 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 after PPV in advanced cancer patients who show higher plasma IL-6 levels [82, 83].

3. OTHER NEW TYPES OF PEPTIDE VACCINES

Recent early phase clinical trials have also demonstrated significant advances in other types of therapeutic peptide-based vaccines [19, 20]. Several new types of peptide-based vaccines are reviewed in this section (Fig. 1).

3.1. Multi-Peptide Vaccine Consisting of CTL and Helper T-Cell Epitopes

Numerous helper T-cell epitopes have been identified from TAA. Since helper T cells are known to play crucial roles in the efficient induction of CTL responses, cancer vaccines, which consist of both HLA class II-restricted helper epitopes recognized by CD4 T cells and class I-restricted CTL epitopes recognized by CD8 T cells, have been developed and clinically tested [84-89]. For example, Kuball *et al.* conducted a phase I study of a multi-peptide vaccine consisting of multiple CTL epitopes from Wilms tumor gene-1 (WT-1), proteinase 3 (Pr3) and mucin 1 (MUC1), and MUC1-helper epitope or pan HLA-DR epitope (PADRE) [84]. Each peptide was formulated separately and injected at a different site. In this study, an increase in PADRE-specific CD4 T cells, which appeared unable to produce IL2, was observed after vaccination, and regulatory T cells were increased, suggesting that helper epitope peptides have the potential to induce not only helper T cells but also regulatory T cells. Krug *et al.* tested the safety and immunogenicity of a WT1 vaccine comprised of four class I and class II-restricted peptides in patients with malignant pleural mesothelioma or NSCLC expressing WT1 [85]. They showed that this multivalent WT1 peptide vaccine induced both CD4 and CD8 T-cell responses in a high proportion of patients with minimal toxicity.

3.2. Multi-Peptide Cocktail Vaccine

If each of multiple peptides are formulated separately and injected at a separate site, the number of peptides employed for vaccination might be limited. One strategy for overcoming this limitation is to generate multi-peptide cocktail vaccines, since one preparation could contain more than 10 different peptides. Although the issue of competition between

individual peptides to bind to HLA molecules on the APCs still remains [46], different types of multi-peptide cocktail vaccines have been developed; vaccines consisting of CTL epitope peptides alone [90, 91] or those of both CTL epitope and helper epitope peptides [86-89].

Barve *et al.* conducted a phase I/II study of a multi-peptide 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 [86]. 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%, with a median overall survival of 17.3 months. One complete response (CR) patient was observed in the total of 63 patients. Slingluff *et al.* conducted a multicenter randomized trial to examine the immunogenicity of a multi-peptide cocktail vaccine containing 12 melanoma-associated HLA class I-restricted peptides (12MP) for $CD8^+$ T cells and tetanus peptide or a mixture of six melanoma-associated helper peptides (6MHP) for $CD4^+$ T cells in the presence or absence of cyclophosphamide pretreatment in 167 patients with resected stage IIB to IV melanoma [87]. However, the combination of 6MHP with 12MP paradoxically reduced the circulating $CD8^+$ T-cell response, and cyclophosphamide pretreatment had no measurable effect on $CD8^+$ or $CD4^+$ responses. Clinical outcome was not improved by adding melanoma-associated helper peptides or by adding cyclophosphamide.

Rammensee and his colleagues also reported a phase I/II trial of a multi-peptide cocktail vaccine, which consisted of 13 synthetic peptides (11 HLA-A*0201-restricted CTL epitopes and 2 helper epitopes derived from prostate tumor antigens) for 19 HLA-$A2^+$ hormone-sensitive prostate cancer patients with biochemical recurrence after primary surgical treatment [88]. The vaccine was well tolerated, and stabilized or slowed down PSA progress in 4 of the 19 patients. The same group also developed another cocktail vaccine, IMA901, which 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, for advanced renal cell cancer [89]. In a randomized phase II trial with a single dose of cyclophosphamide, the number of regulatory T cells was reduced, and immune responses to the vaccine peptides were associated with longer overall survival. A randomized phase III study to determine the clinical benefit of IMA901 is ongoing.

3.3. Hybrid Peptide Vaccine

Peptides used in most clinical trials for peptide-based vaccines possess native amino acid sequences with or without slight modification in anchor amino acids to increase their binding capability to HLA molecules. However, hybrid-type peptide vaccines, which use a new artificial peptide fusing two or more peptides, have been devised. For example, the Ii-Key/HER-2/neu hybrid peptide vaccine, a fusion peptide made up of the Ii-Key 4-mer peptide and HER-2/neu (776-790) helper epitope peptide, has been reported [92, 93]. The Ii/Key 4-mer peptide is the shortest active sequence of the Ii protein, which catalyzes direct charging of MHC class II epitopes to the peptide-binding groove, circumventing the need for intracellular epitope processing [94]. Phase I studies of the Ii-Key/HER-2/neu hybrid peptide vaccine in patients with prostate cancer showed that this vaccine is safe and can induce HER-2/neu-specific cellular immune responses in vaccinated patients [93]. In addition, significant decreases in circulating regulatory T-cell frequencies, plasma HER2/neu, and serum TGF-beta levels were observed.

Nishimura *et al.* reported an artificially synthesized helper/killer-hybrid epitope long peptide (H/K-HELP) of MAGE-A4 cancer antigen [95]. In the first case report, a patient with pulmonary metastasis of colon cancer was vaccinated with MAGE-A4-H/K-HELP in combination with OK432 and Montanide ISA51. There were no severe side effects except for a skin reaction at the injection site. Vaccination with MAGE-A4-H/K-HELP induced MAGE-A4-specific Th1 and Tc1 immune responses and the production of MAGE-A4-specific complement-fixing IgG antibodies. Tumor growth and tumor markers were significantly decreased in this patient.

3.4. Long Peptide Vaccine

The classical types of peptide vaccines have consisted of short epitope peptides with minimal optimal lengths, which are recognized by CTLs or helper T cells in an HLA class I- or class II-restricted manner, respectively. However, direct binding of short peptides to nonspecific cells without a co-stimulatory capacity has been reported to bear the potential to induce tolerance to antigen-specific T cells rather than to induce their activation in some mouse models [39-41]. Therefore, a novel approach using synthetic long peptides, which need to be taken up by professional APCs and processed for presentation by HLA class I and/or class II molecules, has been developed for cancer vaccination, although the efficiency and mechanisms of presentation of exogenous long peptides in human HLA class I remain to be fully elucidated [96]. Synthetic long peptides may contain not only HLA class I-restricted but also HLA class II-restricted epitopes, which can activate helper T cells important for the efficient induction of antigen-specific CTL responses.

Several clinical studies using a pool of multiple synthetic long peptides have been reported, since a mixture of multiple synthetic long peptides is likely to contain multiple HLA class I-restricted and class II-restricted T-cell epitopes, which could be applicable to any patients irrespective of their HLA types [42-45, 97-100]. Melief and his colleagues showed that a vaccine composed of a synthetic long peptide pool derived from high-risk-type human papillomavirus (HPV)-16 E6/E7 oncoproteins successfully induced HPV-specific immune responses [42, 43]. They conducted a phase I study of HPV16 E6 and E7 overlapping long peptides in end-stage cervical cancer patients [42]. Cocktails of nine E6 peptides and/or four E7 peptides covering the entire sequences of E6 and E7 proteins showed a strong and broad T-cell response dominated by immunity against E6 after four subcutaneous administrations with Montanide ISA51 at 3-week intervals. Subsequently, they conducted a phase II study of the same vaccine in patients with HPV-positive grade 3 vulvar intraepithelial neoplasia, which is a chronic disorder caused by HPV [43]. 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%).

The same group also reported a synthetic long peptide vaccine targeted for p53. This p53 synthetic long peptide vaccine (p53-SLP) consisted of 10 synthetic 25-mer to 30-mer long overlapping peptides, spanning amino acids 70–248 of the wild-type p53 protein. In a phase I/II trial of the p53-SLP vaccine in 10 patients with metastatic colorectal cancer, p53-specific T-cell responses were induced in 9 of 10 patients as measured by IFN-γ enzyme-linked immunospot (ELISPOT), proliferation, and cytokine bead arrays [97]. Subsequently, a phase II study of the same vaccine in 20 ovarian cancer patients with recurrent elevation of CA-125 showed that SD, as determined by CA-125 levels and CT scans, was observed in 2 out of 20 patients (10%) as the best clinical response, but no relationship was found between the clinical response and vaccine-induced immunity [44]. IFN-γ–producing p53-specific responses were induced in CD4 T cells, but not in CD8 T cells, in all patients who received four immunizations. The absence of p53-specific CD8 T-cell responses might be attributable to the dominant production of Th2 cytokines by CD4 T cells, which have inhibitory effects on CTL induction. Nevertheless, the combined use of p53-SLP vaccine and a low dose of cyclophosphamide or IFN-α has recently been reported to efficiently induce more IFN-γ–producing p53-specific T cells, suggesting that these combinations may potentiate the immunogenicity of the p53-SLP vaccine [98, 99].

Kakimi *et al.* also conducted a phase I trial of an NY-ESO-1 synthetic long peptide vaccine. A 20-mer peptide spanning from amino acid 91 to 110 of NY-ESO-1, called NY-ESO-1f, which includes multiple epitopes recognized by antibodies and CD4 and CD8 T cells, was administered along with OK-432 and Montanide ISA51 to patients with advanced cancers [100]. Both antigen-specific CD4 and CD8 T-cell responses, as well as antibody responses, were increased in 9 of 10 patients.

3.5. Novel Approach for Targeting Peptides to Professional APCs

The goal of cancer immunotherapy is to induce and amplify functional antigen-specific immune responses in order to develop long-lasting immunological memory specific to tumor cells [101, 102]. However, one hurdle to the use of peptide-based vaccines is that the uptake and/or presentation of vaccine peptides by nonspecific cells, but not by professional APCs, leads to CTL anergy through insufficient stimulation [103]. For efficient priming and activation of antigen-specific CTL through vaccination, sufficient amounts of antigens should be presented to T cells by functionally activated, professional APCs for sufficient periods of time [104-107]. In this respect, a novel delivery system for peptide vaccines remains to be developed.

For example, nanotechnology-based antigen delivery has been developing as a vaccine strategy due to its dose-sparing and prolonged antigen presentation features [108, 109]. In particular, polymeric nanoparticles (NP) have attracted increasing attention as carriers of therapeutic immunogens [110]. Antigen peptides encapsulated in polymeric NP are shown to be directly and specifically delivered to professional APCs via phagocytosis without proteolytic degradation, and efficiently cross-presented to induce strong T-cell immunity, whereas those in solution that are internalized by APCs via macropinocytosis are reported to be poorly presented as peptides in complex with MHC class I molecules on cell surfaces [111, 112]. Indeed, we have demonstrated the feasibility of NP consisting of a biodegradable, biocompatible copolymer, poly(D,L-lactide-co-glycolide) (PLGA) carrying antigenic peptides and a toll-like receptor 4 agonist, monophosphoryl lipid A, to efficiently induce CTL responses against TAA in murine tumor models [113]. To increase the efficacy of peptide-based vaccines, such a novel antigen delivery system remains to be developed and clinically examined.

CONCLUSIONS

In the field of cancer immunology and immunotherapy, excitement and enthusiasm have risen around the latest approvals of immunotherapy-based treatments in various cancer types. However, several issues remain to be addressed in order to achieve further development of cancer vaccines. In particular, in view of the complexity and diversity of tumor cell characteristics and host immune cell repertoires, the selection of vaccine peptides appropriate for individual patients based on the pre-existing host immunity before vaccination could be critical for the efficient induction of beneficial antitumor responses in cancer patients. In a series of clinical trials, we have demonstrated promising results of PPV as a new treatment modality for patients with various types of advanced cancer. Further randomized phase III clinical trials are essential to validate the clinical benefits of PPV. Moreover, novel biomarkers for selecting patients who would benefit most from PPV remain to be addressed.

CONFLICT OF INTEREST

Akira Yamada is an Executive Officer for Green Peptide Company, Ltd. Kyogo Itoh received a research grant from the Green Peptide Company, Ltd. and owns stock in the Green Peptide Company, Ltd.

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