Table 6. Selection of a Gene Classifier for Predicting Short-Term Survival

Training/Test	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Accuracy (%)
Training, n = 40	17/20 (85)	15/20 (75)	17/22 (77)	15/18 (83)	32/40 (80)
Test, n = 13	7/7 (100)	5/6 (83)	7/8 (88)	5/5 (100)	12/13 (92)

value, negative predictive value, and accuracy of 100%, 83%, 88%, 100%, and 92%, respectively, for the prediction of short-term survival (Table 6).

Increase in the Prevaccination Plasma IL-6 Levels in the Patients With Poor Prognosis

Expression of cytokines, chemokines, and growth factors, which may result from proinflammatory and/or anti-inflammatory tumor microenvironments, gives a broad picture of the immunological status of cancer patients.[32-35] We therefore examined the levels of these soluble factors using a bead-based multiplex assay with prevaccination plasma samples from the long-term and short-term survivors. As shown in Figure 3, the plasma levels of proinflammatory cytokine IL-6 were significantly higher in the short-term survivors than in the long-term survivors ($P = .009$). However, the plasma levels of other cytokines, chemokines, or growth factors, including IL-1Rα, IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-α, IFN-γ, TNF-α, G-CSF, GM-CSF, IP-10, RANTES, Eotaxin, MIP-1α, MIP-1β, MCP-1, MIG, VEGF, EGF, HGF, and basic FGF, were not significantly different between the 2 groups (data not shown).

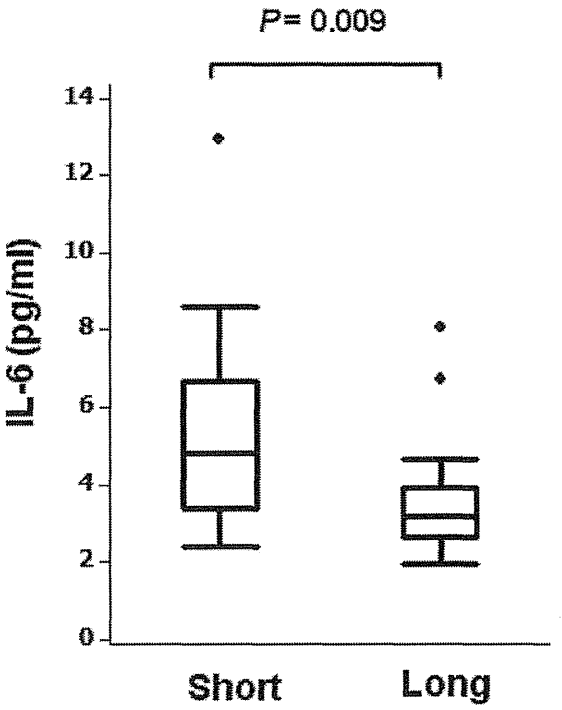

Figure 3. Increase in plasma interleukin (IL)-6 levels in the short-term survivors is shown. The levels of IL-6 assessed by bead-based multiplex assay in prevaccination plasma were compared between the short-term (n = 18) and long-term (n = 18) survivors. Box plots show median and interquartile range (IQR). The whiskers (vertical bars) are the lowest value within 1.5 × IQR of the lower quartile and the highest value within 1.5 × IQR of the upper quartile. Data not included between the whiskers were plotted as outliers with dots. Two-sided *P* value was calculated with Mann-Whitney test.

DISCUSSION

The identification of biomarkers to predict clinical responses to treatment is a challenging but important issue for the development of individualized therapies.[5-8] Although recent advances in high-throughput microarray technology have allowed gene expression profiling for subclassifications of patients in a variety of fields, including organ transplantation and autoimmune diseases,[18-20] little information is available regarding gene expression profiles in peripheral blood of patients treated with immunotherapies. In the current study, to identify promising biomarkers that are predictive of patient prognosis after personalized peptide vaccination, we examined gene expression profiles in PBMCs from 40 advanced castration-resistant prostate cancer patients who showed good or poor prognosis after personalized peptide vaccination. Our DNA microarray analysis in PBMCs identified distinctive genes that were differentially expressed between the long-term and short-term survivors. Interestingly, a statistical prediction model provided a 4-gene classifier that was able to predict patient prognosis with an accuracy of 92% in a validation test, suggesting that the identification of suitable patients for cancer vaccines may be possible with the profiling of a modest number of genes in peripheral blood samples. Because there were no significant differences in the other clinical and pathological

features of the patients enrolled in the current study, except for the number of vaccinations and overall survival, our findings seem to be quite informative for the further development of cancer vaccines.

In the current study, 4 genes, *LRRN3, PCDH17, HIST1H4C*, and *PGLYRP1*, were selected as the best combination for prediction of patient prognosis. *LRRN3* gene encodes a highly conserved transmembrane protein with multiple leucine-rich repeats, which is abundantly expressed in the developing and adult central nervous system. Polymorphisms in this gene were reported to be associated with autism spectrum disorder susceptibility.[36] *PCDH17* is 1 of the cadherin superfamily genes and is expressed predominantly in the nervous system. This molecule was reported to be a tumor suppressor gene candidate in squamous cell carcinomas.[37] *HIST1H4C* gene encodes a member of the histone H4 family, which forms the nucleosome structure of the chromosomal fiber, and may play a central role in transcription regulation, DNA repair and replication, and chromosomal stability.[38] *PGLYRP1* gene encodes a pattern recognition receptor related to innate immunity against bacteria, which is expressed primarily in the granules of granulocytes.[39] Although this information is available from the literature, little is known about the roles of these molecules in immune responses to cancer vaccines. Further studies remain to be done to elucidate them.

One of the most striking features of the differentially expressed genes is that many of the up-regulated genes in both prevaccination and postvaccination PBMCs from the short-term survivors were associated with gene signatures of granulocytes. This may possibly be reflected by the different frequencies of granulocytes in the PBMC fraction purified from peripheral whole blood on density gradient centrifugation using Ficoll-Paque. In healthy donors, normal granulocytes are usually separated from the PBMC fraction on Ficoll-Paque density gradient. However, patients with various types of cancers have been reported to show increased numbers of activated granulocytes in their peripheral blood, which are purified in the PBMC fraction.[40-42] Recently, these abnormal granulocytes have been defined as granulocytic myeloid-derived suppressor cells, which express higher levels of inhibitory molecules, such as ARG1 and inducible nitric oxide synthase,[41,42] and impair the immunological functions of T cells and other immune cells.[43-45] In addition, several studies have recently shown the critical roles for neutrophils, a main subset of granulocytes, in tumorigenesis.[46] Neutrophils have a significant impact on the tumor microenvironment by producing cytokines, chemokines, and other products, such as reactive oxygen species and proteinases, which regulate inflammatory cell activation/recruitment, tumor cell proliferation, angiogenesis, and metastasis.[47-49] For example, recent clinical studies have revealed that the presence of neutrophils in tumors was significantly associated with poor outcomes.[50,51] Unfortunately, because of the limited availability of blood samples, we have not fully characterized the granulocytes that were purified in the PBMC fraction, but it is highly possible that abnormal granulocytes in peripheral blood inhibit beneficial immune responses and lead to poor prognosis after peptide vaccines. The current study might provide a novel treatment approach capable of enhancing the clinical efficacy of cancer vaccines. Recently, chemotherapeutic drugs, such as gemcitabine and 5-fluorouracil, have been shown to selectively eliminate myeloid-derived suppressor cells in mice.[52,53] In addition, targeting of VEGF-mediated signaling using a tyrosine kinase inhibitor, sunitinib, has been reported to block expansion of $CD15^{+}CD14^{-}$ granulocytic myeloid-derived suppressor cells in patients with renal cell cancers.[54] It would thus be possible that accompanying treatments with such chemotherapeutic or molecularly targeted drugs before providing cancer vaccines suppress the gene signatures related to poor prognosis and improve patient outcomes after personalized peptide vaccination.

In addition to the granulocyte-related genes, other interesting genes were also differentially expressed between the long-term and short-term survivors. For example, leukocyte-associated immunoglobulin-like receptor 2 (*LAIR2*), a member of the immunoglobulin superfamily, was down-regulated in the prevaccination PBMCs of short-term survivors. Although not well studied, this molecule has been suggested to function as a proinflammatory mediator by suppressing the homologous immune inhibitor, leukocyte-associated immunoglobulin-like receptor 1 (*LAIR-1*), which is present on several types of mononuclear leukocytes.[55] In addition, another noticeable finding is that several erythroid-specific genes, such as hemoglobin families (*HBQ1, HBM, HBD), ALAS2, GYPE, EPB42, HP*, and *ERAF*, were up-regulated in the postvaccination PBMCs of short-term survivors. The precise roles of these differentially expressed genes in immune responses to cancer vaccines need to be determined.

Interestingly, when the gene expression profiles in PBMCs were compared between before and after personalized peptide vaccination, many of the differentially

expressed genes in prevaccination and/or postvaccination PBMCs, including granulocyte-related and erythroid-related genes, were up-regulated after personalized peptide vaccination in the short-term survivors, but not in the long-term survivors. This finding may be explained by the possibility that induction of granulocyte and erythroid gene signatures may be prevented by personalized peptide vaccination in the long-term survivors.

It should also be noted that the levels of the proinflammatory cytokine IL-6 in prevaccination plasma were significantly elevated in the short-term survivors. IL-6 is a multifunctional cytokine that regulates various aspects of immune responses, acute phase reactions, and hematopoiesis. In particular, IL-6 has been reported to be deeply involved in inflammation associated with cancer development and progression.[34] There have been many studies describing the correlation between IL-6 levels and prognosis in various types of cancers, including prostate cancer.[56-59] Interestingly, IL-6 has been also shown to rapidly generate myeloid-derived suppressor cells from precursors that are present in murine and human bone marrow or PBMCs, in the presence of other cytokines such as GM-CSF,[60,61] although in the current study, the expression levels of plasma IL-6 were not well correlated with expressions of granulocyte-related genes in the microarray analysis (data not shown). Although the role of IL-6 in the immune responses to cancer vaccines still remains to be clarified, it is possible that the blockage of IL-6 signaling would be beneficial for enhancing the therapeutic efficacy of cancer vaccines.

To the best of our knowledge, this is the first study to characterize gene expression profiles in peripheral blood and thereby identify biomarkers for predicting clinical outcomes after peptide vaccines. Our findings suggest that the widely available gene expression profiling in peripheral blood may permit future development of molecular-based personalized immunotherapies through discrimination between patients with good and poor prognoses. Although our experimental approaches were not novel, the ability to predict patient prognosis on the basis of relatively simple assays with easily available peripheral blood samples would be of importance. It may be possible that the current study would provide important information for defining eligibility and/or exclusion criteria for personalized peptide vaccination in castration-resistant prostate cancer patients. Nevertheless, because this is a retrospective study with a limited number of patients, all of whom received personalized peptide vaccination, clinical utility of the identified gene signatures and gene classifier needs to be confirmed in future larger-scale, prospective trials conducted in defined patient populations receiving or not receiving personalized peptide vaccination. In addition, the gene expression profiles identified in the current study remain to be verified by using other, independent methods for mRNA and/or protein quantification.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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COMMENTARY

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Personalized peptide vaccination

A novel immunotherapeutic approach for advanced cancer

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

Keywords: peptide vaccine, personalized vaccine, cytotoxic T lymphocytes, advanced cancer, biomarker, inflammation

Abbreviations: PPV, personalized peptide vaccination; CTL, cytotoxic T lymphocytes; CRPC, castration-resistant prostate cancer; FDA, food and drug administration; MST, median survival time; HR, hazard ratio; CI, confidence interval

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Introduction

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

Rationale for Personalized Selection of Vaccine Antigens in Individual Cancer Patients

A large number of tumor-associated antigens have been identified by several different approaches, including cDNA expression cloning, serologic analysis of recombinant cDNA expression libraries (SEREX), and reverse immunological approach.[8] Although the number of cancer vaccine candidates is becoming almost limitless, antigens currently employed

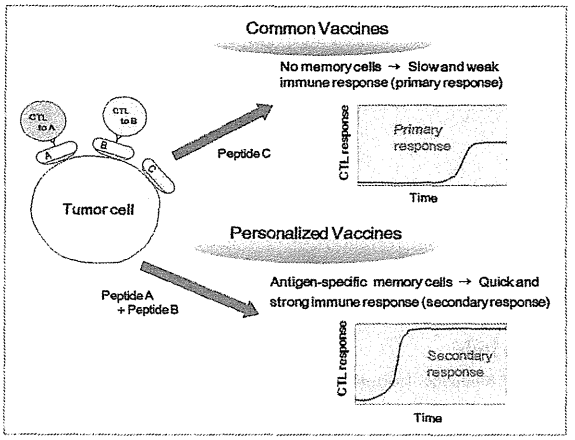

Figure 1. Personalized vaccines are more promising than common vaccines. Personalized antigens can induce quick and strong secondary immune responses, whereas common antigens without immunological memory induce slow and weak primary immune responses.

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

Nevertheless, in most of current clinical trials of therapeutic cancer vaccines, common antigens are employed for vaccination independently of immunological status of patients. Patients, who have immunological memory to vaccine antigens, are expected to show quick and strong immune responses to them. In contrast, patients with no immunological memory against vaccine antigens would take more time for development of effective anti-tumor immune responses, because several rounds of repeated vaccinations might be required to prime antigen-specific naive T cells to functional effector cells (**Fig. 1**). In such situations, vaccinations could not easily provide clinical benefits, especially in advanced cancer patients, who show a relatively quick disease progression. Moreover, immune responses induced by inadequate vaccines that are non-specific to tumor cells may not only be ineffective for tumor control, but also erode pre-existing immunity.[9] Based on the current paradigm that the size and composition of the adaptive immune system are limited and that individual immune cells are constantly competing each other in the limited space, inadequate vaccination may have negative consequences for the hosts by suppressing pre-existing beneficial memory cells specific to tumors and/or infections, which might result in acceleration of cancer progression or early death in vaccinated patients.[10] Considering these issues, it would be quite reasonable that vaccine antigens should be selected based on the pre-existing immunological status in each patient.

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

PPV as a Novel Immunotherapeutic Approach

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

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

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

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

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

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

In the clinical trials of PPV conducted during the past several years, we have shown promising results in various types of cancers.[12,13,16,18,19] **Table 1** shows the clinical responses in 500 advanced cancer patients who received PPV from October 2000 to October 2008.[16] The best clinical response assessed in 436 evaluable patients were partial response (PR) in 43 patients (10%), stable disease (SD) in 144 patients (33%) and progressive disease (PD) in 249 patients (57%), with a median overall survival of 9.9 mo. Of note, as shown in **Figure 2**, a recently conducted phase II randomized clinical trial of PPV for 57 CRPC patients demonstrated that patients receiving PPV in combination with low-dose estramustine phosphate (EMP) showed a significantly longer progression-free [median survival time (MST), 8.5 vs. 2.8 mo; hazard ratio (HR), 0.28 (95% confidence interval (CI), 0.14–0.61); p = 0.0012] and overall survival [MST, undefined vs. 16.1 mo; HR, 0.30 (95% CI, 0.10–0.91); p = 0.0328] than those receiving standard-dose EMP alone.[18] In addition, PPV was also conducted in an early phase clinical trial of patients with recurrent or progressive glioblastoma multiforme, one of the most aggressive brain tumors, with median overall survival of 10.6 mo.[19] Based on these promising results, randomized phase III trials are currently underway in CRPC and glioblastoma. To prove clinical benefits of PPV for accelerating cancer vaccine development, further randomized phase III trials would also be recommended in other different types of cancers.

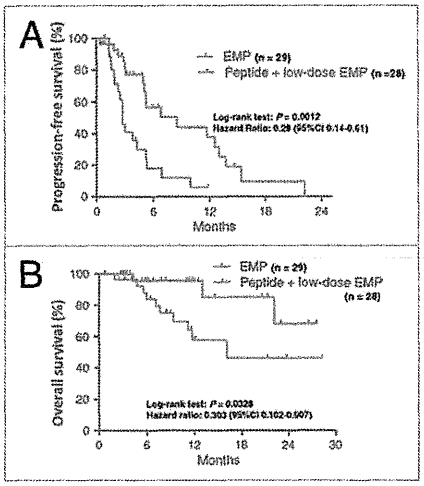

Figure 2. Progression-free and overall survival in patients with castration-resistant prostate cancer using personalized peptide vaccination. Kaplan-Meier curves of progression-free (A) and overall survival (B) in patients treated with personalized peptide vaccination plus low-dose estramustine phosphate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

Lymphocyte Counts, Increased Humoral Responses to the Vaccine Antigens, and Inflammatory Factors as a Biomarker for PPV

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

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

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

To identify biomarkers useful for selecting appropriate patients before vaccination, we further addressed pre-vaccination prognostic markers in patients with several different types of advanced cancers who underwent PPV. In CRPC treated with PPV (n = 40), a comprehensive study of soluble factors and gene expression profiles by microarray analysis demonstrated that higher IL-6 level and granulocytic myeloid-derived suppressor cells (MDSC) in the peripheral blood before vaccination were closely associated with poorer prognosis.[24] In patients with refractory non-small cell lung cancer (n = 41), multivariate Cox regression analyses showed that higher C-reactive protein (CRP) level before vaccination was a significant predictor of unfavorable overall survival (HR = 10.115, 95% CI = 2.447–41.806, $p = 0.001$).[25] In addition, in refractory biliary tract cancer patients (n = 25), higher IL-6 and lower albumin levels before vaccination were significantly unfavorable factors for overall survival [HR = 1.123, 95% CI = 1.008–1.252, $p = 0.035$; HR = 0.158, 95% CI = 0.029–0.860, $p = 0.033$; respectively].[26] Collectively, these findings have demonstrated that less inflammation may contribute to better responses to PPV, suggesting that evaluation of the inflammatory factors before vaccination could be useful for selecting appropriate cancer patients for PPV. Based on these findings, an early phase clinical trial is currently underway to show whether the blockage of IL-6-mediated inflammatory signaling with a humanized anti-IL-6 receptor monoclonal antibody, tocilizumab, would be beneficial for enhancing the immune and/or clinical responses of PPV.[27]

Conclusions

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

Disclosure of Potential Conflicts of Interest

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

Acknowledgements

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Review Article

Next-generation peptide vaccines for advanced cancer

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

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

Multivalent long peptide vaccines

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

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

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

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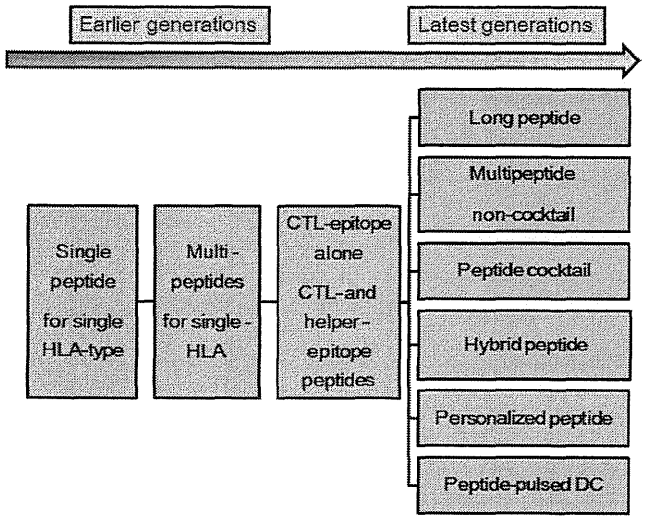

Fig. 1. Transition of peptide vaccine development for advanced cancer. DC, dendritic cells.

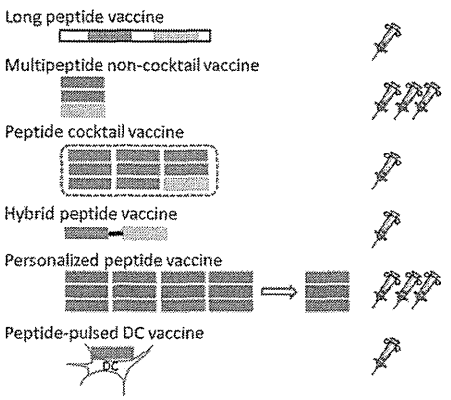

Fig. 2. Various types of latest generation peptide vaccines. The number of syringes indicates that of the final preparation for injection. Green, CTL-epitopes; orange, helper-epitopes. DC, dendritic cells.

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

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

Multipeptide vaccines consisting of CTL- and helper-epitopes

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

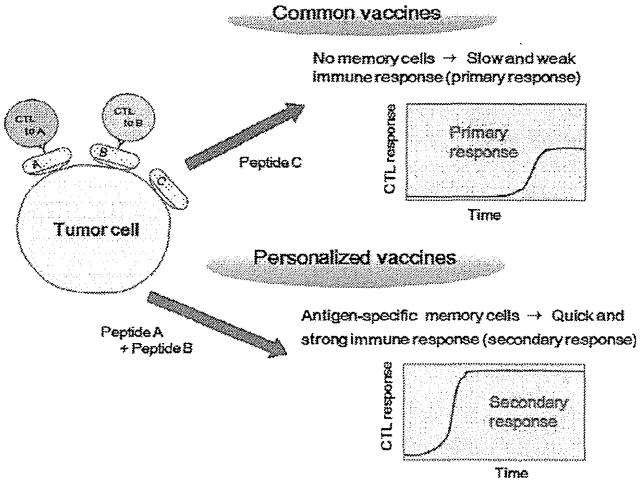

Fig. 3. Personalized peptide vaccine. In the classical type of vaccine, peptides derived from tumor-specific or overexpressed antigens are used as vaccine peptides and often mismatched to the pre-existing immunity of patients. In personalized peptide vaccines, appropriate peptides for vaccination are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

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

Peptide cocktail vaccines

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

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

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

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

A3sup, A3 super type; CR, complete response; CRPC, castration-resistant prostate cancer; G3, grade 3; HLA, human leukocyte antigen; M, months; MST, median survival time; Not ref., not referred; NSCLC, non-small-cell lung cancer; PI, phase I clinical trial; PII, phase II clinical trial; PC, prostate cancer; PD, progressive disease; PFS, progression-free survival; PR, partial response; RCC, renal cell carcinoma; Ref., reference; SCLC, small-cell lung cancer; SD, stable disease.

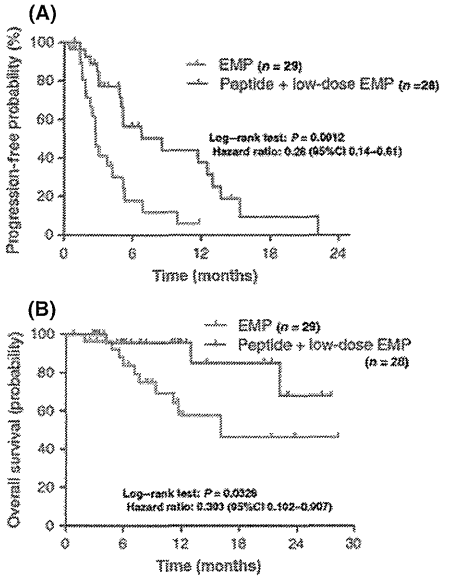

Fig. 4. Randomized phase II trial of personalized peptide vaccine (PPV) plus low-dose estramustine phosphate (EMP) versus standard-dose EMP in patients with castration-resistant prostate cancer. Patients were randomized into groups receiving either PPV plus low-dose EMP (280 mg/day) or standard-dose EMP (560 mg/day). (A) Duration of progression-free survival in the first treatment. (B) Overall survival of patients treated with PPV plus low-dose EMP and standard-dose EMP. CI, confidence interval.

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

Hybrid peptide vaccines

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

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

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

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

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of MHC class II epitopes to the peptide-binding groove, circumventing the need for intracellular epitope processing, and the shortest active sequence of the Ii protein is the Ii/Key peptide.[24] Holmes *et al.*[22] and Perez *et al.* [23] reported the results of phase I studies of the Ii-Key/HER -2/neu hybrid peptide vaccine in patients with prostate cancer. Significant decreases in circulating regulatory T cell frequencies, plasma HER-2/neu, and serum transforming growth factor-β levels were observed when compared with the native HER-2/neu (776–790) peptide vaccination.

Takahashi and colleagues developed a hybrid peptide of a helper-epitope and CTL-epitope of MAGE-A4.[25] The phase I study of the vaccine was carried out in patients with advanced cancers who were vaccinated with MAGE-A4-H/K-HELP combined with OK432 and Montanide ISA51. In a case report, there were no severe side-effects except for a skin reaction at the injection site. The vaccine induced MAGE-A4-specific Th1 and Tc1 immune responses and the production of MAGE-A4-specific complement-fixing IgG antibodies. Tumor growth and the carcinoembryonic antigen tumor marker were significantly decreased in the final diagnosis.

Personalized peptide vaccines

Virtually all prevaccination patients already have a weak immunity to cancer cells. However, the characteristics of cancer cells and of the immunological status against cancers differ widely among patients, even among those with the same histological types of cancer and identical HLA types. One of the reasons for the low clinical efficacies of the earlier generations of peptide vaccines might be a mismatch between the vaccine peptides and pre-existing immunity to the cancer cells. We therefore attempted to optimize the vaccine peptides so that they were appropriately matched to the pre-existing immunity of each patient (Fig. 3). There are two ways to detect pre-existing immunity, detection of CTL-precursors and detection of IgG in the peripheral blood. The PBMCs were cultured with vaccine peptide panels and the CTL responses to each peptide were measured. The second method is to detect IgG antibodies to the vaccine peptide panels. It is well known that the production of the IgG class of antibodies requires T-cell help. Therefore, the presence of a specific IgG indicates the presence of helper T cells. We carried out a series of clinical trials using personalized peptide vaccines (PPVs) for advanced cancer patients.[26–50] In this PPV formulation, appropriate peptide antigens for vaccination are screened and selected from a panel of vaccine candidates in each patient, based on pre-existing host immunity as mentioned above. Currently, we use 31 HLA class I-restricted peptide candidates, which were identified from a variety of tumor-associated antigens mainly through the cDNA expression cloning method with tumor-infiltrating T-lymphocyte lines, 12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for HLA-A3 supertype (A3, A11, A31, or A33), and 4 peptides for HLA-A26. The safety and potential immunological effects of these vaccine candidates have been shown in previous clinical studies.[26,27] A maximum of four peptides, which were selected based on the results of HLA typing and the pre-existing immune responses specific to each of the 31 different vaccine candidates, were injected s.c. with Montanide ISA51 weekly or bi-weekly.

Currently, we evaluate the pre-existing immune responses to vaccine candidates by B cell responses, but not by T cell responses, as the performance characteristics, such as the sensitivity and reproducibility, of the current T cell assays are far from satisfactory. In contrast to these drawbacks inherent to T cell assays, B cell assays have more potential for screening and/or monitoring antigen-specific immune responses even to HLA class I-restricted peptides. For example, we have recently published several papers describing the clear correlations between clinical benefits and antigen-specific B cell responses measured by IgG antibody production in patient plasma after vaccination. Notably, the multiplex bead-based Luminex technology that we have developed for monitoring B cell responses allow simple, quick, and highly reproducible high-throughput screening of IgG responses specific to large numbers of peptide antigens with a tiny amount of plasma.

In the clinical trials of PPV carried out during the past decade, we have shown promising results in various types of cancers.[26–50] Table 1 shows the summary of the immunological and clinical responses in 460 advanced cancer patients who received PPV. The best clinical responses assessed in the 436 evaluable patients were a partial response in 43 patients (10%), stable disease in 144 patients (33%), and progressive disease in 249 patients (57%), with a median overall survival of 9.9 months. Of note, a recent phase II randomized clinical trial of PPV for 57 castration-resistant prostate cancer patients showed that patients receiving PPV in combination with low-dose estramustine phosphate (EMP) showed a significantly longer progression-free (median survival time, 8.5 months *vs* 2.8 months; hazard ratio, 0.28 [95% confidence interval, 0.14–0.61]; $P = 0.0012$) and overall survival (median survival time, undefined *vs* 16.1 months; hazard ratio, 0.30 [95% confidence interval, 0.1–0.91]; $P = 0.0328$) than those receiving standard-dose EMP alone, suggesting the feasibility of this combination therapy (Fig. 4).[44] In addition, PPV was also used in an early phase clinical trial of patients with recurrent or progressive glioblastoma multiforme, one of the most aggressive brain tumors, with a median overall survival of 10.6 months.[47] Based on these promising results, randomized phase III trials are currently underway in glioblastoma. To prove the clinical benefits of PPV for accelerating cancer vaccine development, further randomized phase III trials would also be recommended in other types of cancers.

Peptide-pulsed dendritic cell vaccines

Many clinical trials of dendritic cell (DC)-based vaccinations using autologous DC and tumor-associated antigen peptides have been carried out to assess the ability of these vaccines to induce clinical responses in cancer patients.[51–54] Rahma *et al.*[54] carried out a comparative study of DC-based vaccine versus non-DC-based authentic peptide vaccine. Twenty-one advanced ovarian cancer patients were divided two groups: arm A received a p53 CTL-epitope peptide with Montanide with IL2; arm B received the same peptide-pulsed DCs with IL2. The median progression-free survival and overall survival were 4.2 (arm A) *i* 8.7 (arm B) months and 40.8 (arm A) versus 29.6 (arm B) months, respectively. This study suggests that the simple peptide vaccination and labor-consuming DC-based vaccination therapy are similarly effective.

Conclusion

Many investigators have attempted to develop more effective cancer vaccines, and in this review we discussed the resulting progress in the latest generation of peptide vaccines. The pros and cons of each type of vaccine are shown in Table 2. Each study used different adjuvants, cytokines, and/or other combination therapies with different doses. Moreover, the individual peptides themselves had different immunological and clinical potency as well as different amino acid sequences. Therefore, it is very hard to conclude that one type of vaccine was more efficient than another. The role of immune checkpoint molecules, such as CTLA-4 and programmed cell death-1, on antitumor immunity was clarified, and promising results have been reported in the clinical trials using combination therapies

with peptide vaccines and immune checkpoint blockades.[55–57] Further randomized phase III trials would be essential to prove the clinical benefits of these vaccine therapies, including immune checkpoint blockade combination therapies.

Disclosure Statement

The author Akira Yamada is an Executive Officer for Green Peptide Company, Ltd.

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In vitro induction of specific CD8$^+$ T lymphocytes by tumor-associated antigenic peptides in patients with oral squamous cell carcinoma

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ABSTRACT

The aim of this study was to clarify candidate peptides for peptide-based specific immunotherapy of patients with oral squamous cell carcinoma (SCC). Thirteen peptides were examined for *in vitro* induction of peptide-specific CD8$^+$ T lymphocyte (CD8$^+$TL) activity in peripheral blood mononuclear cells from 35 patients with oral SCC. A correlation between the induction ability of CD8$^+$TL and *in vivo* immune response of host was carried out immunohistochemically in 23 patients. Peptide-specific activities of CD8$^+$TL for at least one peptide were detectable in 21/35 patients (60.0%). The potent peptides were SART-1_{690} in 9/35 (25.7%), SART-2_{93}, and ART4_{75} in 7/35 (20.0%), respectively. In the 9 patients with SART-1_{690}-specific activity, the whole of activities was significantly inducible for more number of other peptides compared to that in 26 patients without the activity ($P = 0.035$). Cellular responses in 7 patients with SART-1_{690}-specific activity were significantly stronger than those in 16 patients without the activity ($P = 0.027$). Furthermore, the number of CD3$^+$ T cells around the SCC was also significantly different between the 2 groups of patients ($P = 0.041$). In conclusion, SART-1_{690}, SART-2_{93}, and ART4_{75} could be applicable as peptide-based specific immunotherapies for the majority of patients with oral SCC.

1. Introduction

Recent advances in molecular biology and tumor immunology have facilitated identification of antigenic peptides recognized by HLA class I-restricted cytotoxic T lymphocytes (CTLs) against melanomas [1,2] and epithelial cancers [3–6], thereby opening the door to peptide-based immunotherapies for cancer patients. In fact, as cancer vaccines in clinical trials, some immunotherapies have produced significant tumor regression in patients with melanomas [7–9]. These tumor-rejection peptides are thus expected to offer a new tool for specific immunotherapy of cancer patients.

Though combination therapy including surgery, radiotherapy, and chemotherapy for oral squamous cell carcinoma (SCC) has maintained the overall 5-year survival rate at about 80% [10], these modalities cannot completely control the prognosis of the rest 20% patients with oral SCC. In such an urgent condition, immunotherapy for oral SCC has been focused on as a fourth modality for the treatment. In oral SCC, tumor-infiltrating lymphocytes (TILs) are commonly observed [11], suggesting that the host immunological surveillance could definitely recognize the SCC. Therefore, specific immunotherapy with the tumor-rejection antigens is also expected as a new treatment modality for patients with oral SCCs [12,13]. Recently, several SCC-associated antigenic peptides have been identified, that are recognized by cytotoxic CTLs, such as SART-1 [14,15], 2 [16], 3 [17], cyclophilin B (CyB) [18], ART4-derived peptides [19], and p56^{lck} (Lck) [20]. However, little is known about the usefulness of these candidate peptides for a peptide-based specific immunotherapy of oral SCC.

To verify simply the superiority of the peptides, the *in vitro* induction of peptide-specific CD8$^+$ T lymphocyte (CD8$^+$TL) has been commonly carried out. The advantages exhibit direct measurement of CD8$^+$TL activity and handling with large samples. Therefore, the first aim of this study was to clarify suitable antigenic peptides reacted by CD8$^+$TLs in peripheral blood mononuclear cells (PBMCs) from patients with oral SCCs *in vitro*. The second aim was to immunohistochemically elucidate a correlation between the induction ability of CD8$^+$TL and *in vivo* immune response of host.

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2. Materials and methods

2.1. Patients and samples

Serologic HLA class I typing of PBMCs was performed in 35 patients with oral SCC who had been referred to the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital. All patients consented prior to the procedure. Among them, 9 and 26 patients were determined to be HLA-A24-homozygous and -heterozygous, respectively, and a total of the 35 HLA-A24$^+$ patients were enrolled in this study. Heparinized peripheral blood samples were obtained from them at the same time of each biopsy prior to any anticancer treatment, and the PBMCs were prepared by Ficoll-Conray density gradient centrifugation and used for *in vitro* induction of peptide-specific CD8$^+$TLs. Tumor biopsy specimens from 23 of the 35 HLA-A24$^+$ patients were immediately placed in an embedding medium (OCT compound; Miles, Elkhart, IN, USA), frozen, and then used for immunohistochemical staining. The diagnosis was confirmed by histopathologic examinations with H&E staining, and the grades of differentiation, and the degrees of lymphocytic responses were determined according to the criteria of the WHO [21], and Willen et al. [22] respectively.

2.2. Immunohistochemical staining

For the immunohistochemical analysis, the streptoavidin–biotin methodology was used. Four-mm-thick sections were cut from frozened materials, mounted on glass slides, and then were air-dried. The sections were immersed in methanol containing 0.3% hydrogen peroxidase for 10 min to block the endogenous peroxidase activity, and then were incubated with 10% normal rat serum for 30 min to eliminate any non-specific binding. Thereafter, the sections were incubated with primary monoclonal antibodies for 60 min, then with biotinylated rat antimouse antibodies for 10 min, followed by staining with avidin–biotinylated peroxidase complex (Histofine *SAB-PO kit; Nichirei, Tokyo, Japan). The primary antibodies used were* A9 (anti-CD3, mouse IgG2a, 1:1000 dilution, kindly provided by Dr. S.M. Fu, University of Virginia, Charlottesville, VA). All procedures were performed at room temperature. Negative controls were treated in the same way, but the primary antibodies were replaced by normal mouse IgG or IgM. The stained sections with less than 25% reactive cells were considered to be negative. The cut-off points were established at 25%, 50% and 75% and defined 25–49% (weak), 50–74% (moderate), and more than 75% (strong) reactive cells as +, ++, and +++, respectively.

2.3. Peptides

The synthesized peptides used in this study are listed in Table 1. These peptides were purchased from Sigma Genosys (Hokkaido, Japan) and the purity levels were >90%. All of the peptides, except for Epstein-Barr virus (EBV)-derived peptide, were previously reported to be encoded by tumor-rejection antigens and to induce HLA-A24$^+$-restricted CD8$^+$TLs from PBMCs of the patients with SCC, with antigenic specificity for SCC [14,16,18–20,23]. EBV-derived peptide was used as a positive control for *in vitro* induction of CD8$^+$TLs.

2.4. In vitro induction of CD8$^+$TLs by antigenic peptides

The methods used for *in vitro* CD8$^+$TL induction by antigenic peptides and the estimation of CD8$^+$TL activity have been described previously [14,15,24]. In brief, the PBMCs (1×10^5/well) were incubated with 10 mM of each peptide in U-bottom-type 96-well plates that contained 200 ml of culture medium [45% RPMI-1640, 45% AIM-V medium (Invitrogen, Carlsbad, CA), 10% FCS (Equitech Bio, Ingram, TX), 100 U/ml recombinant IL-2 (Shionogi, Co., Osaka, Japan), 0.1 mM MEM nonessential amino acid solution (GIBCO-BRL)]. Half of the medium was removed and replaced with fresh medium that contained the appropriate peptide (20 mM) every 3 days for up to 13 days to keep the density in each well. As a negative control, fresh medium that lacked the peptide was replaced. On the 13th day of the culture, 24 h after the last stimulation, the cells were harvested, washed 3 times, and then tested for the ability to produce interferon-γ (IFN-γ) in response to C1R-A2402 cells that were preloaded with either the corresponding peptide or without peptide (negative control) in HLA-A24$^+$ PBMCs. The target cells (C1R-A2402, 1×10^4/well) were pulsed with each peptide (10 mM) or without peptide (negative control) for 2 h, and the effector cells (1×10^5/well) were then added to each well to a final volume of 200 ml. After incubation for 18 h at effector to target cell (E/T) ratios of 40:1, 20:1, 10:1, 5:1, and 1:1 in quadruplicate assays, the supernatants (100 ml) were collected, and the ability of IFN-γ production were measured by ELISA (Japan Immunoresearch Laboratories Co., Gunma, Japan). The threshold of sensitivity was 5 pg/ml. All experiments were performed in quadruplicate. The two-tailed Student's *t*-test was employed for the statistical analyzes. Detectable levels of CD8$^+$TLs were adjudged as positive when the mean value of IFN-γ production by the peptide-stimulated PBMCs in response to a corresponding peptide was significantly ($P < 0.05$) higher than that in response to no peptide.

Table 1
Antigenic peptides used for *in vitro* CD8$^+$TL induction.

Antigenic peptide	Sequence
SART-1_{690}	EYRGFTQDF
SART-2_{93}	DYSARWNEI
SART-2_{161}	AYDFLYNYL
SART-2_{899}	SYTRLFLIL
SART-3_{109}	VYDYNCHVDL
SART-3_{315}	AYIDFEMKI
CyB_{84}	KFHRVIKDF
CyB_{91}	DFMIQGGDF
Lck_{208}	HYTNASDGL
Lck_{486}	TFDYLRSVL
Lck_{488}	DYLRSVLEDF
ART_{413}	AFLRHAAL
ART_{475}	DYPSLATDI
EBV-derived	TYGPVFMCL

2.5. Statistical analysis

The statistical significance of differences between the groups was determined by Fisher's exact probability test and two-tailed Studen'ts *t*-test. *P* values less than 0.05 were considered to be significant.

3. Results

PBMCs from the 35 HLA-A24$^+$ patients with oral SCC were examined for their reactivity to all 13 kinds of tumor-associated antigenic peptides. The PBMCs were stimulated *in vitro* with each peptide for 13 days and the induction of peptide-specific CD8$^+$TL activity was estimated by IFN-γ production in response to a corresponding peptide, as performed in our previous report [13]. Representative results in Fig. 1 shows that the increased IFN-γ production was dependent upon the increased number of effector cells. These results indicate that peptide-specific CD8$^+$TLs were satisfactorily induced. Table 2 shows mean values of IFN-γ production at E/T ratio of 10:1 in quadruplicate assays, and summarizes frequencies of induction of peptide-specific CD8$^+$TL activity from the PBMCs with each peptide. The background IFN-γ production in response to no peptide has been subtracted from the values given. CD8$^+$TL activities were adjudged to be positive and underlined, if the mean value of the peptide-stimulated PBMCs in response to the corresponding peptide was significantly higher than that in response to no peptide. The EBV-derived peptide induced detectable peptide-specific CD8$^+$TL activity in all of the patients. Overall, peptide-specific CD8$^+$TL activities for at least one tumor-associated peptide were detectable in 21 (60.0%) of the 35 patients examined for all 13 peptides, with a mean of 1.7 peptides/one patient (range of 0–7 peptides). The profiles of the CD8$^+$TL-inducible peptides varied among the patients. In contrast, the PBMCs from 14 (40.0%) of the 35 patients showed no detectable levels of CD8$^+$TL activity in response to any tumor-associated peptides. The most potent peptide to induce peptide-specific CD8$^+$TL activities was SART-1_{690} and the CD8$^+$TL activity was inducible in 9/35 patients (25.7%). SART-2_{93} and $ART4_{75}$ were also potent peptides and the CD8$^+$TL activities were both induced in 7/35 patients (20.0%).

In the 9 patients with detectable SART-1_{690}-specific CD8$^+$TL activity (patients 1, 2, 3, 4, 5, 10, 18, 19 and 20), peptide-specific CD8$^+$TL activities were inducible significantly for more number of other peptides (Mean ± SD: 4.3 ± 1.7), compared with that (Mean ± SD: 0.7 ± 0.9) in 26 patients with no detectable SART-1_{690}-specific activity ($P = 0.035$; two-tailed Student's *t*-test). In contrast, CD8$^+$TL activities induced by EBV-derived peptide, which was used as a positive control, were detectable in all of the 35 patients, and were not significantly different between the 2 groups of patients (data not shown). These results suggest that the capability of *in vivo* immune response against tumor cells may be different between these 2 groups of patients.

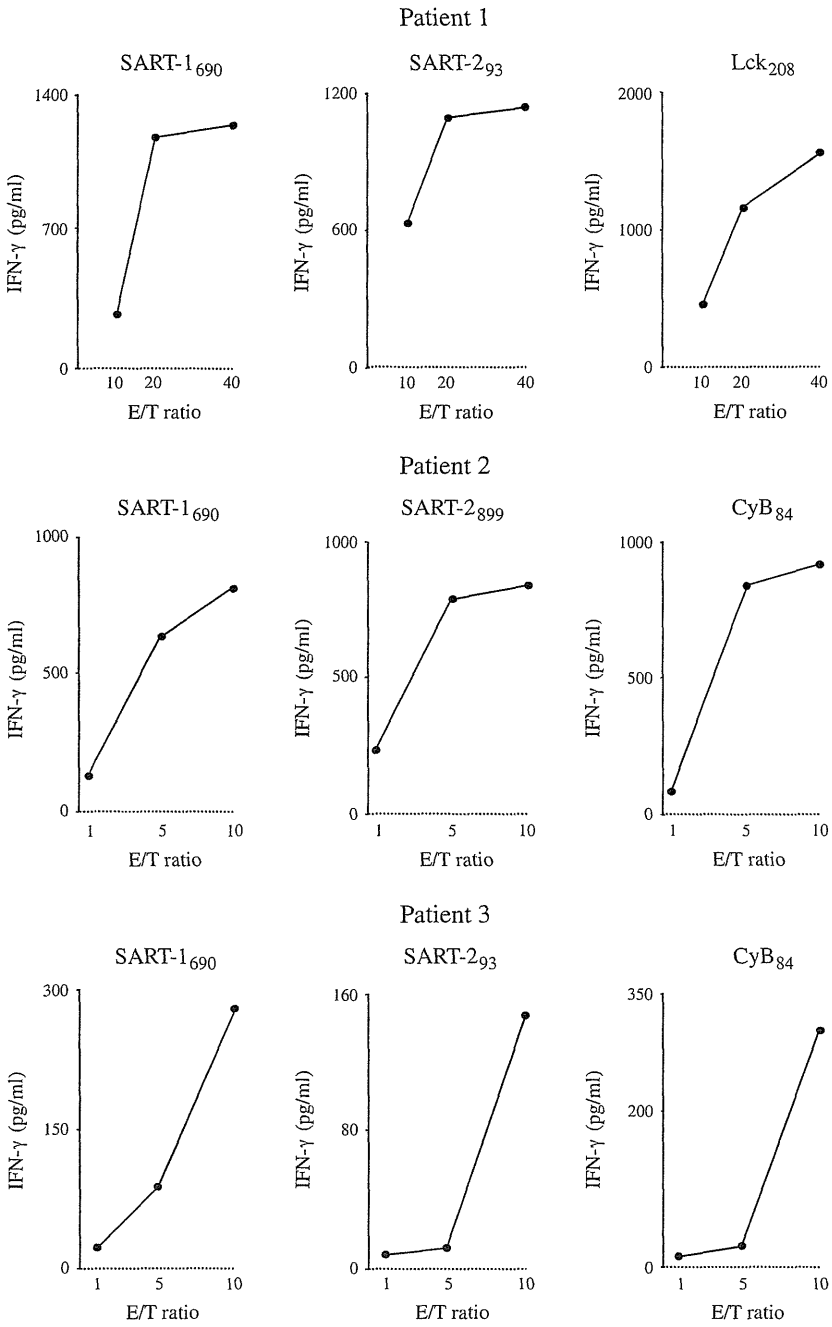

Fig. 1. Dose dependence of $CD8^+$TLs activities induced by antigenic peptides. PBMCs (1×10^5/well) from patient 1, 2, and 3 were stimulated with 13 kinds of antigenic peptides (Table 1) for 13 days and tested for their ability to produce IFN-γ in response to C1R-A2402 cells at day 13. The peptide-specific $CD8^+$TL activities were examined at different E/T ratios in quadruplicate assays. Mean values of quadruplicate assays are shown. Similar results were obtained from an additional 18 patients.

We thus compared histologic findings, such as cellular response and differentiation of SCC, between the 2 groups of patients, as shown in Table 3. This comparison was performed in 23 patients in whom immunohistochemical analysis with tumor biopsy specimens could be carried out. Interestingly, cellular responses in 7 patients with the detectable SART-1_{690}-specific activity (patients 1, 2, 3, 4, 5, 18 and 19) were significantly stronger than those in 16 patients with no detectable SART-1_{690}-specific activity ($P = 0.027$; Fisher's exact probability test), although the grades of differentiation of SCC were not significantly different. Furthermore, the number of $CD3^+$ T cells around SCC was also significantly different between the 2 groups of patients ($P = 0.041$; Fisher's exact probability test). Taken together, it was strongly suggested that immunogenecity of tumor cells or responsiveness of host immune system to tumor cells totally differs between the 2 groups of patients.

4. Discussion

Although peptide-specific $CD8^+$TL activities could be detected in 21/35 (60.0%) patients with oral SCC, only 13 peptides were avail-