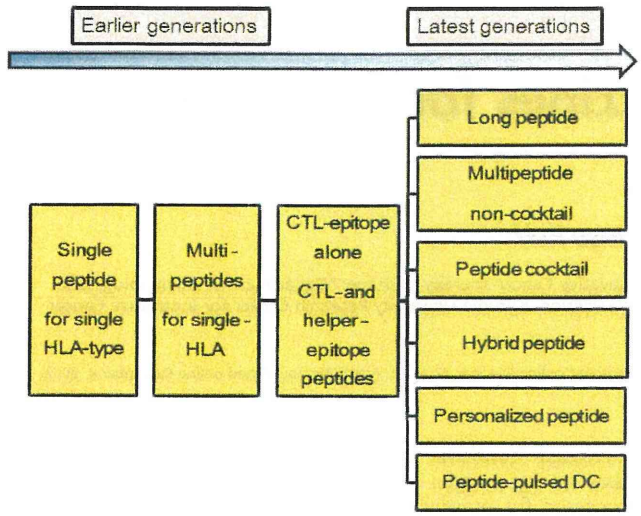


**Fig. 1.** Transition of peptide vaccine development for advanced cancer. DC, dendritic cells.

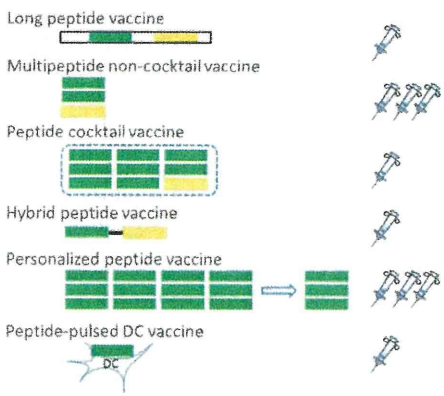


**Fig. 2.** Various types of latest generation peptide vaccines. The number of syringes indicates that of the final preparation for injection. Green, CTL-epitopes; orange, helper-epitopes. DC, dendritic cells.

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

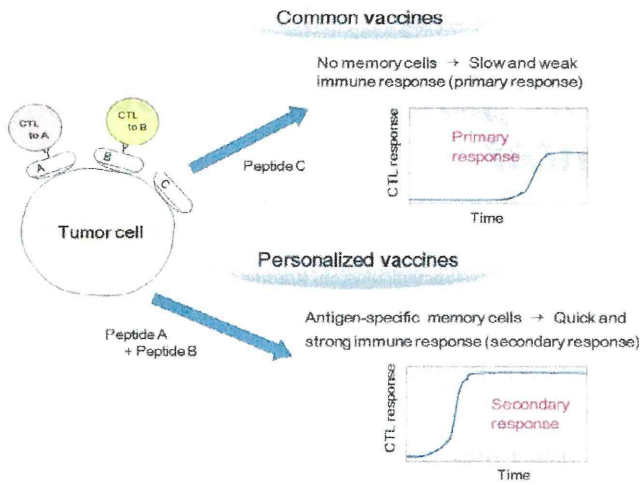


**Fig. 3.** Personalized peptide vaccine. In the classical type of vaccine, peptides derived from tumor-specific or overexpressed antigens are used as vaccine peptides and often mismatched to the pre-existing immunity of patients. In personalized peptide vaccines, appropriate peptides for vaccination 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.

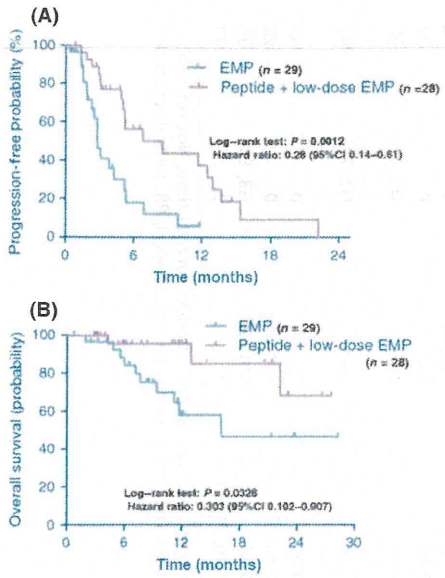


**Fig. 4.** Randomized phase II trial of personalized peptide vaccine (PPV) plus low-dose estramustine phosphate (EMP) versus standard-dose EMP in patients with castration-resistant prostate cancer. Patients were randomized into groups receiving either PPV plus low-dose EMP (280 mg/day) or standard-dose 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**

| | Pros | | | | | Cons | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Vaccine type | 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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# A phase II trial of personalized peptide vaccination in castration-resistant prostate cancer patients: prolongation of prostate-specific antigen doubling time

Masanori Noguchi[1,2*], Fukuko Moriya[2], Shigetaka Suekane[2], Rei Ohnishi[2], Satoko Matsueda[3], Tetsuro Sasada[3], Akira Yamada[4] and Kyogo Itoh[3]

## Abstract

**Background:** Cancer vaccine is one of the attractive treatment modalities for patients with castration-resistant prostate cancer (CRPC). However, because of delayed immune responses, its clinical benefits, besides for overall survival (OS), are not well captured by the World Health Organization (WHO) and Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Several surrogate markers for evaluation of cancer vaccine, including prostate-specific antigen doubling time (PSADT), are currently sought. The purpose of this study was to assess prospectively the PSA kinetics and immune responses, as well as the efficacy, safety, and biomarkers of personalized peptide vaccination (PPV) in progressive CRPC.

**Methods:** One hundred patients with progressive CRPC were treated with PPV using 2–4 positive peptides from 31 candidate peptides determined by both human leukocyte antigen (HLA) class IA types and the levels of immunoglobulin G (IgG) against each peptide. The association between immune responses and PSADT as well as overall survival (OS) was studied.

**Results:** PPV was safe and well tolerated in all patients with a median survival time of 18.8 months. Peptide-specific IgG and T-cell responses strongly correlated with PSADT ($p < 0.0001$ and $p = 0.0007$, respectively), which in turn showed correlation with OS ($p = 0.018$). Positive IgG responses and prolongation of PSADT during PPV were also significantly associated with OS ($p = 0.001$ and $p = 0.004$) by multivariate analysis.

**Conclusions:** PSADT could be an appropriate surrogate marker for evaluation of the clinical benefit of cancer vaccine. Further randomized trials are needed to confirm these results.

**Trial registration:** UMIN000001850

**Keywords:** Prostate-specific antigen doubling time, Personalized peptide vaccine, Prostate cancer, Surrogate marker, Overall survival

## Background

Changes in serum prostate-specific antigen (PSA) can reflect the burden of disease and clinical benefit in patients with castration-resistant prostate cancer (CRPC) with cytotoxic chemotherapy or hormonal agents known to kill tumor cells; these changes can have practical utility by providing and updating prognostic information on an individual patient over time [1-4]. As observed in many clinical trials, however, immunotherapy can induce novel patterns of antitumor responses distinct from those of chemotherapy [5]. For example, an autologous dendritic-cell-based vaccine (sipuleucel-T) is known to improve survival without having an impact on early PSA decline [6], whereas docetaxel's improvement in overall survival (OS) correlates for the most part with a PSA decline within the first 3 months of therapy [7,8]. Thus, interpreting PSA decline in the context of novel immunotherapy must be

* Correspondence: noguchi@med.kurume-u.ac.jp
[1]Clinical Research Division of the Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan
[2]Departments of Urology, Kurume University School of Medicine, Kurume, Japan
Full list of author information is available at the end of the article

carried out with caution on the basis of the mechanism of action, and may also depend on the time of sampling [9].

Personalized peptide vaccine (PPV) uses multiple peptides based on the pre-existing immunity. Under PPV treatment, each patient with human leukocyte antigen (HLA)-class IA types positive was tested for their immunological reactivity to 31 different peptides capable of inducing T-cell responses. The 31 peptides were derived from a number of tumor associated antigens: PSA, prostatic acid phosphatase (PAP), prostate-specific membrane antigen (PSMA), multidrug resistance protein and a variety of other epithelial tumor antigens. We previously demonstrated that PPV was safe and improved OS with immune responses in phase I, I/II, and II clinical trials in patients with CRPC [10-16]. However, it was not addressed whether PSADT could be an appropriate surrogate marker for evaluation of the clinical benefit of cancer vaccine. To address this, we evaluated data from a phase II clinical trial for CRPC using PPV.

## Methods

### Patient Eligibility

Eligibility required a histological diagnosis of prostate adenocarcinoma and progressive disease (PD) defined as at least two consecutive increases in PSA, new metastatic lesion on radionuclide bone scan, or progressive tumor lesions on cross-sectional imaging, despite adequate androgen ablative therapy. Patients showed positive IgG

**Table 1 Peptide candidates for personalized peptide vaccination**

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

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

**Table 2 Patient characteristics**

| Characteristics | Patients (N = 100) No. |
|---|---|
| Age, years | |
| Median | 69 |
| Range | 51-92 |
| ECOG performance status | |
| 0 | 91 |
| 1 | 9 |
| HLA typing | |
| A24 | 66 |
| A2 | 21 |
| A3 supertype | 11 |
| A26 | 2 |
| Baseline PSA, ng/ml | |
| Median | 29.8 |
| Range | 0.2-2481 |
| PSADT, months | |
| Median | 2 |
| Range | 0.3-36+ |
| Lymphocyte, 1300/μL | |
| Low | 41 |
| High | 59 |
| CRP, 3 μg/mL | |
| Low | 53 |
| High | 47 |
| SAA, 8 μg/mL | |
| Low | 27 |
| High | 76 |
| IL6, 2.4 pg/mL | |
| Low | 84 |
| High | 16 |
| Gleason score | |
| ≤7 | 34 |
| ≥8 | 57 |
| Unknown | 9 |
| Site of metastasis | |
| no | 14 |
| Bone only | 33 |
| Bone and nodal/organ | 40 |
| Nodal/organ | 13 |
| Prior chemotherapy | |
| (-) | 60 |
| (+) | 40 |

*Abbreviations*: PPV, personalized peptide vaccination; ECOG, Eastern Cooperative Oncology Group; HLA, human leucocyte antigen; PSA, prostate-specific antigen; PSADT, PSA doubling time; CRP, C-reactive protein; SAA, serum amyloid A; IL6, interleukin 6.

responses to at least two of the 31 different candidate peptides (Table 1). Any number of previous hormonal therapies was allowed. Patients were required to wait at least four weeks for entry into the study after the completion of prior radiation therapy, chemotherapy, or a change in hormonal therapy. Other inclusion criteria included age ≥ 20 years; Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1; life expectancy of at least 12 weeks; positive status for HLA-A2, -A24, -A3 supertype (–A3, -A11, -A31, and -A33), or -A26; adequate hematologic, hepatic, and renal function; and negative status for hepatitis virus B and C. Exclusion criteria included an acute infection; a history of severe allergic reactions; pulmonary, cardiac, or other systemic diseases; and other inappropriate conditions for enrollment as judged by clinicians.

### Study design and treatment

This was a single institution, single arm, open-label, phase II study. The endpoints of this study were primarily safety and feasibility of PPV in patients with CRPC. Secondary endpoints were to assess the PSA kinetics and immune responses. In addition, we identified potential factors for predicting OS and selecting suitable patients for this treatment. This study protocol was approved by Kurume University Ethical Committee. Written informed consent was obtained from all patients before any study procedures.

In this study, 31 peptides, whose safety and immunological effects had been confirmed in previously conducted clinical studies [10-18], were employed for vaccination [12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for the HLA-A3 supertype (A3, A11, A31, or A33), and 4 peptides for HLA-A26] (Table 1). All peptides were prepared under conditions of Good Manufacturing Practice using a Multiple Peptide System (San Diego, CA). The selection of 2 to 4 peptides for vaccination to each patient was based on HLA typing and high titer level of peptide-specific IgG to candidate peptides. Each of the selected peptides was mixed with incomplete Freund's adjuvant (Montanide ISA-51VG; Seppic, Paris, France) and emulsified in the 5 ml plastic syringe, and a maximum of four peptides of 1.5 ml emulsion (3 mg/peptide) were injected subcutaneously into the lateral thigh area once a week for 6 weeks. The

peptides were re-selected according to peptide-specific IgG levels at every cycle of 6 vaccinations and administered at 2-, 3-, or 4-week intervals until withdrawal of consent or unacceptable toxicity.

### Assessment of clinical activity

Patients were monitored at each visit by history and physical examinations. Serum PSA test and routine laboratory studies were performed every 6 vaccinations for any adverse effects. Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI-CTCAE Ver3).

All patients underwent relevant radiologic studies and bone scans every 6 months or at the progression of symptoms. PD was defined as radiographic progression evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) criteria [19] or clinical progression.

To assess the PSA response for each patient, percent PSA change from baseline was calculated for each phase of the study (pre- and during vaccination). In addition, PSA doubling time (PSADT) was calculated using all serum PSA values for a specified period, and using a minimum of three PSA values by the formula $\log_2/b$, where b denotes the least square estimate of the linear regression model of the log-transformed PSA values on time. For analytical purposes, negative PSADT estimates and high positive PSADT estimates (>36 months) were censored at 36 months.

To investigate biomarkers for OS that may allow patient selection and prediction of a response to PPV, serum amyloid A (SAA), C-reactive protein (CRP), and interleukin (IL)-6 in plasma at baseline were additionally examined by enzyme-linked immunosorbent assay (ELISA), respectively.

### Measurement of humoral and T-cell responses specific to the vaccinated peptides

To study the humoral responses specific to the vaccinated peptides, peptide-specific IgG levels were measured by a Luminex system (Luminex, Austin, TX), as reported previously [20]. If the total titers of selected peptide-specific IgG in any cycles of post-vaccination plasma were more than 2-fold higher than those in the pre-vaccination plasma, the changes were considered to be a positive response.

Although T-cell subsets using flowcytometry was not analyzed in this study, T-cell responses specific to the vaccinated peptides were evaluated by IFN-γ ELISPOT assay using peripheral blood mononuclear cells (PBMCs), as reported previously [18]. Peptide-specific T-cell responses were evaluated by the differences between the numbers of spots per $10^5$ x PBMCs in response to the vaccine peptides and those to the control peptide at pre- and 6th vaccination; at least 2-fold more spots at the 6th vaccination than at pre-vaccination was considered positive.

### Statistical analysis

All patients who received more than 6 vaccinations were considered evaluable for tumor response, and all patients entered were included in the survival analysis. Data were

**Table 3 Adverse events during peptide vaccination**

| | Grade 1 | Grade 2 | Grade 3 | Total |
|---|---|---|---|---|
| Injection site reaction | 73 | 24 | 13 | 43 |
| Constitutional symptoms | | | | |
| Bone pain | 16 | 14 | 13 | 43 |
| Appetite loss | 29 | 5 | 1 | 35 |
| Fatigue | 23 | 11 | 0 | 34 |
| Edema peripheral | 10 | 3 | 0 | 10 |
| Blood/bone marrow | | | | |
| Lymphocytopenia | 17 | 13 | 5 | 35 |
| Anemia | 7 | 7 | 16 | 30 |
| White blood cell count decreased | 6 | 6 | 5 | 17 |
| Laboratory | | | | |
| Hypoalbuminemia | 27 | 13 | 0 | 40 |
| ALP increased | 20 | 8 | 6 | 34 |
| AST increased | 24 | 4 | 1 | 29 |
| Hyponatremia | 24 | 1 | 0 | 25 |
| ALT increased | 13 | 2 | 1 | 16 |
| Blood triglycerides increased | 10 | 2 | 0 | 12 |
| Creatinine increased | 6 | 1 | 2 | 8 |

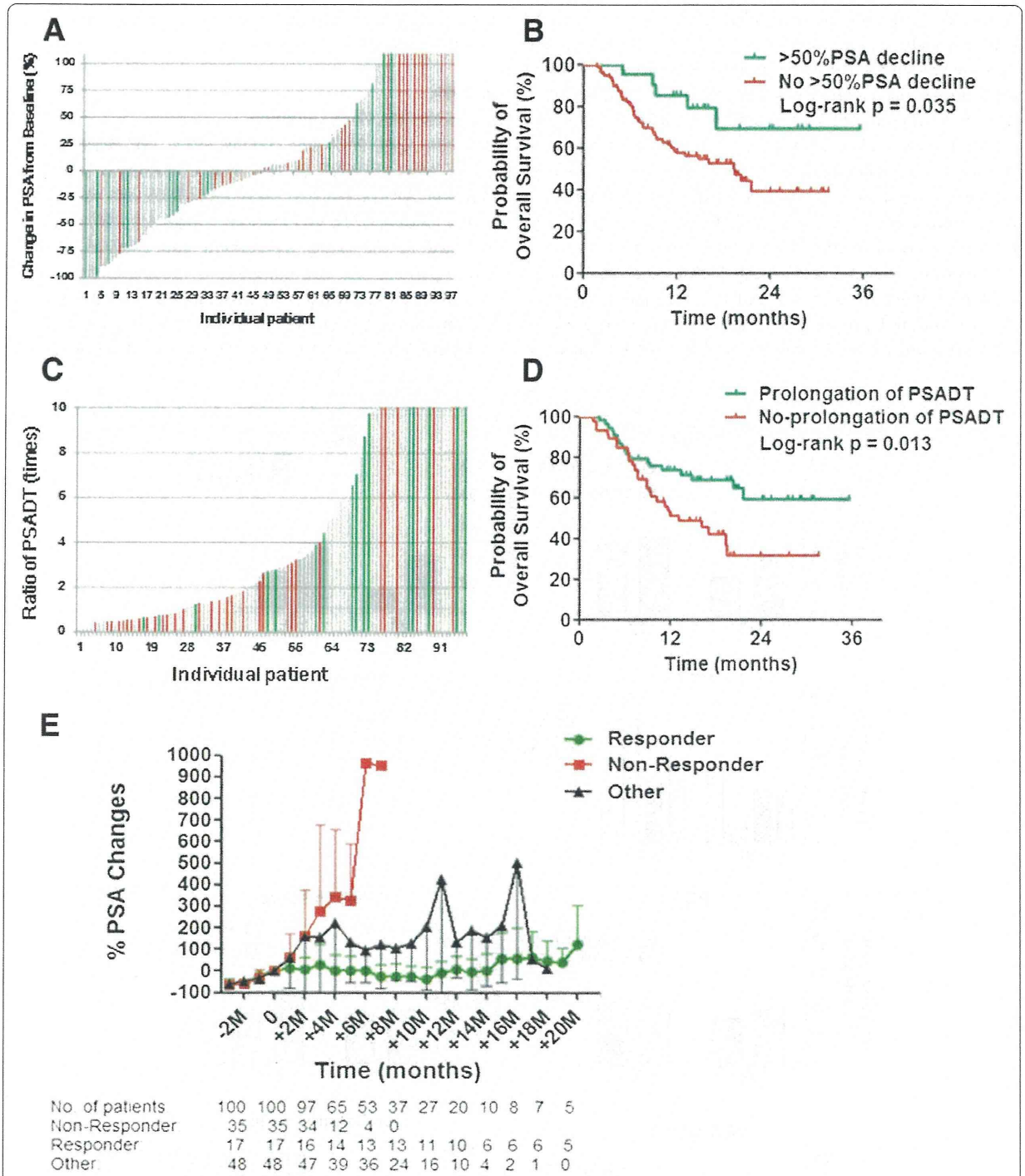


**Figure 1 PSA kinetics and overall survival. (A)** Waterfall plot showing the maximal PSA changes (%) from baseline during personalized peptide vaccination (PPV) at any time point. **(B)** Overall survival by >50% PSA decline. **(C)** The ratio of PSADT changes for each patient pre- and during PPV is plotted. The ratio of PSADT changes was calculated by dividing PSADT during treatment by pre-treatment PSADT. A ratio greater than 2 indicates prolongation of PSADT. **(D)** Overall survival by prolongation of PSDT. **(E)** Longitudinal average PSA changes (%) before and during PPV. Green histograms: Responder group (alive for more than 20 months). Red histograms: Non-responder group (death within 12 months). Gray histograms: Other group.

analyzed at the end of November, 2012 using commercially available computer software. The Student's t-test and the chi-square test were used to compare quantitative and categorical variables, respectively. Survival was calculated from the date of first treatment until the date of any cause of death. Patients lost to follow-up were censored at the last known date of survival. The Kaplan-Meier method was used to estimate actuarial survival curves, and groups were compared using a log-rank test. Cox proportional hazards regression model was used for univariate and multivariate analyses to identify factors that had a significant impact on survival. All baseline parameters in the survival and proportional hazards regression analysis were analyzed as dichotomous variables using median or cut-off values. A two-sided significance level of 5% was considered statistically significant.

## Results

### Characteristics of the patients

Between April 2009 and August 2011, 100 patients with CRPC were enrolled in this trial at Kurume University Hospital. All 100 patients received at least one vaccination with a median of 16 vaccinations (range, 1 to 40) and were included in the safety assessment and survival analysis. Three patients did not complete 6 vaccinations (1 cycle) and were excluded from the assessment of PSA response and immune responses. The reason for these failures to complete 6 vaccinations was withdrawal of consent. The

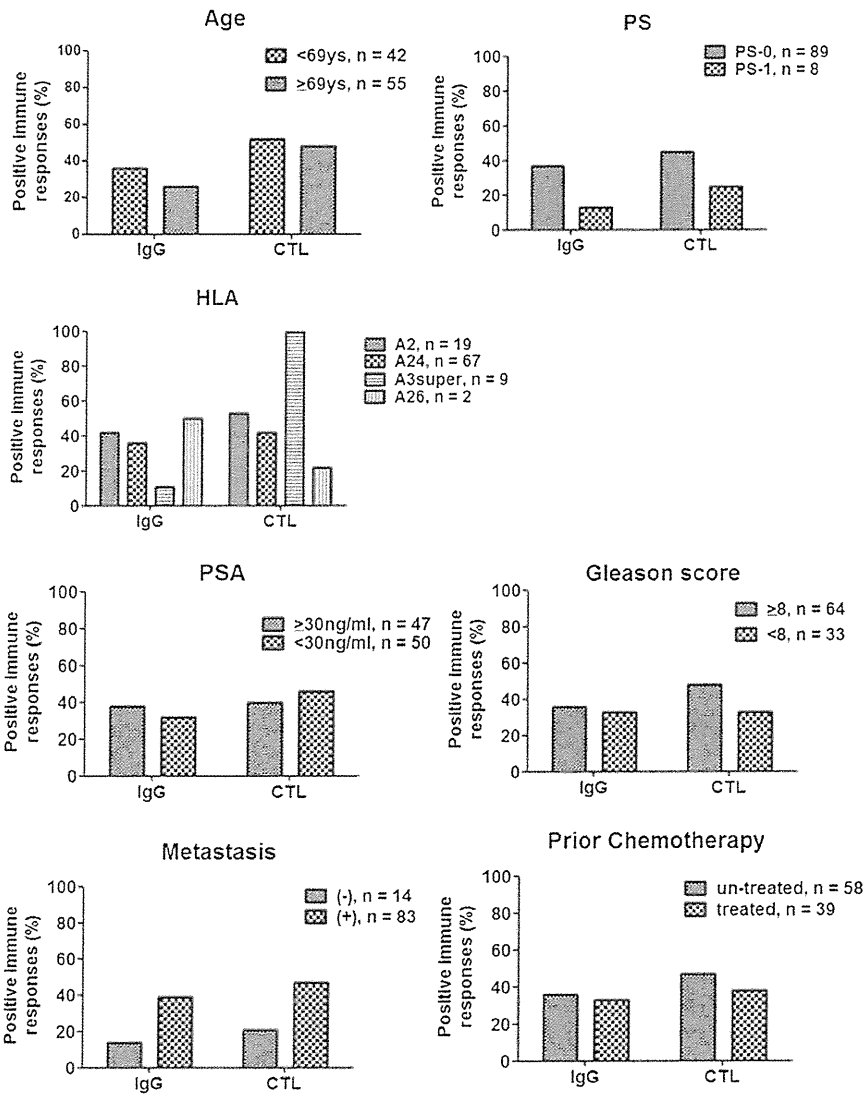


**Figure 2** Positive immune responses of IgG and CTL based on baseline characteristics.

median age of participants was 69 years (range, 51 to 92 years), and the ECOG performance status was 0 in 91of the patients and 1 in the remaining 9. The median PSA and pre-vaccination PSADT at the entry to the study was 29.8 ng/ml (range, 0.2 to 2481 ng/ml) and 2 months (range, 0.3 to 36+ months), respectively. Fifty-seven patients had a Gleason score of ≥ 8 and 86 patients had metastasis. All patients had experienced progression after androgen deprivation therapy as an initial or secondary therapy. Forty patients had received docetaxel based chemotherapy with a median cycle of 6.5 as a third line treatment. Baseline patient characteristics are shown in Table 2.

### Adverse events

The overall toxicities are shown in Table 3. The most frequent adverse events were local redness and swelling at injection sites, bone pain, hypoalbuminemia, lymphocytopenia, appetite loss, fatigue, increased ALP, and anemia, which were grade 1 or 2 in most cases. There were no grade 4 toxicities and no treatment-related deaths. A total of 51 grade 3 toxicities including anemia, bone pain, increased ALP, lymphocytopenia, decreased white blood cells, increased creatinine, injection site reaction, and increased AST and ALT were observed during the study. All of these severe adverse events were concluded to be not directly associated with the vaccinations, but with cancer progression or other causes by the independent safety evaluation committee in this trial.

### Clinical outcome

Forty-eight (49%) patients exhibited some decrease in PSA from baseline, ranging from 1.9% to 99.6% (Figure 1A). Confirmed ≥50% PSA decline at any point during PPV was observed in 21 patients (22%), with a median time of 4 months to ≥50% PSA decline and a median duration of ≥50% PSA decline of 3 months. Delayed PSA response was observed. Patients with ≥50% PSA decline during PPV showed longer survival than remaining patients ( $p = 0.035$) (Figure 1B). The median estimated PSADT pre- and during PPV were 2 and 3.89 months, respectively. Fifty-four (56%) patients displayed at least 2-fold increase over the pre-treatment PSADT (range, 2.1- to 75-fold), and these patients with a prolongation of PSADT showed longer survival than patients without a prolongation of PSADT ($p = 0.013$) (Figure 1C and D). To compare the difference in PSA responses with clinical outcomes, patients were divided into three groups: responder group with survival longer than 20 months after PPV, non-responder group with death within 12 months after PPV, and another group with the remaining patients. Average% PSA changes in the responder group were significantly lower than those in the non-responder group at 2 to 5 months ($p < 0.005$) and those in the other group at 5 to 10 months ($p < 0.005$) during the PPV. In addition, average% PSA changes in the responder group showed a trend of PSA plateau. Average% PSA changes from baseline among three groups before and during PPV are shown in Figure 1E.

There was no complete response or partial response in terms of measurable disease. The median time to disease progression, as defined by clinical and/or radiologic criteria, was 10.9 months (95% CI, 6 to 19 months). At the time of analysis with a median follow-up of 18 months (95% CI, 14.1 to 24 months), 64 deaths had occurred. Median survival time was 18.8 months (95% CI, 14.9 to 28.6 months) in all patients. Median survival time in chemotherapy naive patients and in patients after docetaxel chemotherapy were 21.6 months and 11.6 months, respectively.

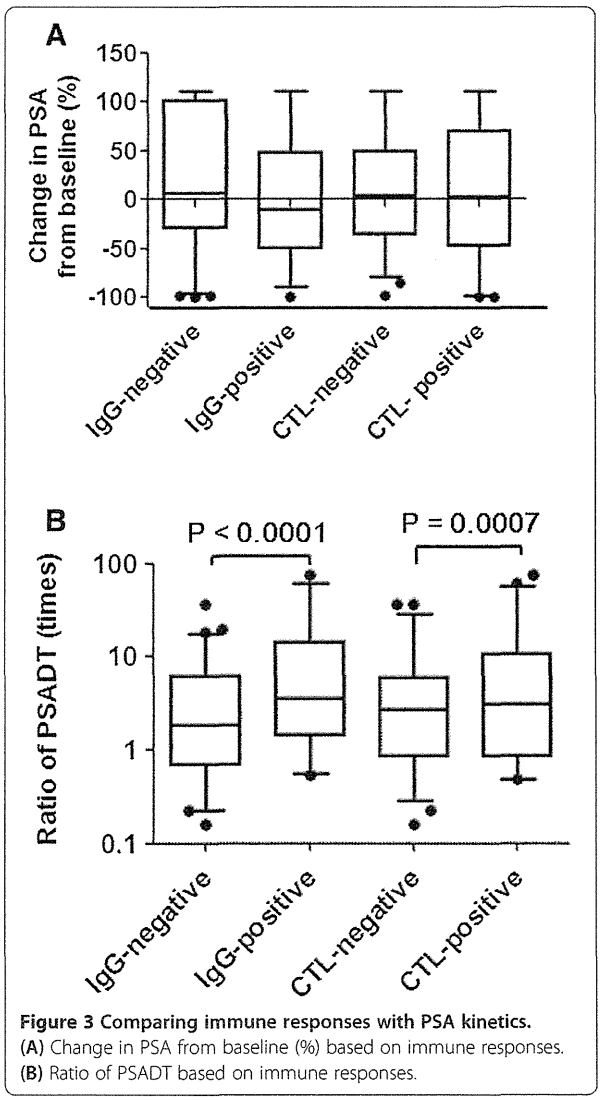


**Figure 3 Comparing immune responses with PSA kinetics.** **(A)** Change in PSA from baseline (%) based on immune responses. **(B)** Ratio of PSADT based on immune responses.

### Immunological response

The number of selected peptides were 4 peptides in 62 patients, 3 peptides in 17 patients and 2 peptides in 21 patients at the first screening. Same peptide at the first screening were only selected in 29 of 97 (30%) patients at second screening and in 10 of 66 (15%) patients at the third screening, remaining patients received at least 1 different peptide during the study. The most frequently selected peptides were Lck486 (40 patients), CypB129 (31 patients), PAP213 (24 patients), SART2-93 (21 patients), PSA248 (20 patients), Lck488 (17 patients) and WHSC2-123 (16 patients) at the first screening. All 31 peptides were selected at any screening in the study.

Total IgG responses specific to the vaccinated peptide were augmented in 42 of 97 (43%) patients, 62 of 66 (94%) patients, 36 of 36 (100%) patients, 16 of 16 (100%) patients, and 7 of 7 (100%) patients at the 6th, 12th, 18th, 24th, and 30th vaccinations, respectively. Finally, positive IgG responses during PPV were observed in 76/97 (79%) patients. PBMCs from 97 patients were available for IFN-γ Elispot assay at the pre- and 6th vaccination. Peptide-specific T-cell responses were detectable in 42 patients (43%) at the 6th vaccination. There was no obvious correlation between IgG and CTL responses. Positive immune responses of both IgG and CTL based on baseline characteristics including age, PS, HLA typing, PSA, Gleason score, presence of metastasis and prior chemotherapy are shown in Figure 2. There was no difference in positive immune responses among baseline characteristics. In comparing immune responses with PSA kinetics, although average PSA changes did not correlate with immune responses, average ratio of PSADT was significantly higher in patients with positive IgG (8 vs. 4, $p < 0.0001$) and CTL (8.8 vs. 6.1, $p = 0.0007$) responses (Figure 3).

### Survival analysis

Cox proportional hazards regression analysis was performed to determine factors that would predict disease death (Table 4). Univariate Cox analysis showed that good performance status ($p < 0.0001$), positive IgG response ($p < 0.0001$), low CRP ($p = 0.012$), prolongation of PSADT ($p = 0.018$), low PSA ($p = 0.004$), prior chemotherapy status ($p = 0.037$), positive T-cell response ($p = 0.039$), and presentation of ≥50% PSA decline ($p = 0.046$) were significantly associated with survival.

The factors showing $p$ less than 0.05 in the univariate analysis were included in multivariate analysis of the model. Finally, positive IgG response ($p = 0.001$) and prolongation of PSADT ($p = 0.004$) during PPV, as well as baseline good performance status ($p = 0.004$), low CRP levels ($p = 0.006$), and low PSA levels ($p = 0.008$), were significantly favorable factors for OS (Table 4).

## Discussion

As observed in several clinical trials, immunotherapy can induce novel patterns of antitumor responses distinct from those of chemotherapy, which are consequently not captured by the WHO or RECIST criteria [5]. On the other hand, there is debate regarding the utility of PSA changes, especially with immunotherapy, and the PSA Working Group 2 has advocated using radiographic progression-free survival as a preferred endpoint for phase

**Table 4 Cox proportional hazards regression analysis of association between potential factors and death after PPV in the 100 CRPC patients**

| Factors | Cut-offs[a] | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|---|
| | | p value | Hazard ratio | 95% CI | p value | Hazard ratio | 95% CI |
| IgG response | Positive vs. negative | <0.0001 | 0.19 | 0.101-0.355 | 0.001 | 0.272 | 0.125-0.592 |
| ECOG performance status | 0 vs. 1 | <0.0001 | 0.073 | 0.031-0.174 | 0.004 | 0.179 | 0.056-0.569 |
| CRP | Low (<3000 ng/mL) vs. high | 0.012 | 0.461 | 0.252-0.842 | 0.006 | 0.389 | 0.199-0.759 |
| PSADT | Increase (2 times) vs. no | 0.018 | 0.477 | 0.258-0.881 | 0.004 | 0.357 | 0.176-0.725 |
| PSA | Low (<30 ng/mL) vs. high | 0.004 | 0.407 | 0.221-0.749 | 0.008 | 0.361 | 0.171-0.762 |
| Prior chemotherapy | Untreated vs. treated | 0.037 | 0.536 | 0.298-0.962 | 0.329 | 0.695 | 0.335-1.445 |
| T-cell response | Positive vs. negative | 0.039 | 0.51 | 0.269-0.967 | 0.273 | 0.679 | 0.340-1.357 |
| >50% PSA decline | Positive vs. negative | 0.046 | 0.387 | 0.152-0.984 | 0.553 | 0.733 | 0.263-2.042 |
| Number of lymphocytes | High (>1300/μL) vs. low | 0.054 | 0.562 | 0.313-1.009 | - | - | - |
| IL6 | Low (<2.4 pg/mL) vs. high | 0.057 | 0.491 | 0.236-1.021 | - | - | - |
| Pts. age | Low (<69 years) vs. high | 0.186 | 0.666 | 0.364-1.218 | - | - | - |
| Gleason score | Low (<8) vs. high | 0.623 | 1.162 | 0.637-2.128 | - | - | - |
| SAA | Low (<8 μg/mL) vs. high | 0.709 | 0.875 | 0.433-1.767 | - | - | - |

Of the 100 men, 64 died.

[a]Lymphocyte, PSA, and patient age are based on median values.

*Abbreviations*: PPV, personalized peptide vaccination; CRPC, castration-resistant prostate cancer; CI, confidence intervals; ECOG, Eastern Cooperative Oncology Group; PSA, prostate-specific antigen; PSADT, PSA doubling time; CRP, C-reactive protein; SAA, serum amyloid A; IL6, interleukin 6.

II trials [21]. Others have argued that changes in PSADT may be a marker of drug effect, understanding that shorter PSADT corresponds to worse prognosis and, thus, a favorable change in PSADT suggests drug activity [22,23]. However, clinical trials of recently developed drugs, such as sipuleucel-T [6], cabazitaxel [24], and abiraterone acetate [25], for the treatment of progressive CRPC patients did not analyze the usefulness of PSADT as a surrogate marker of response in CRPC patients. In the current study, we attempted careful and stringent collection of multiple PSA values in order to calculate PSADT changes before and during PPV accurately. While delayed PSA responses were observed, we did see a statistically significant increase in PSADT. Importantly, patients with prolongation of PSADT showed statistically longer survival ($p = 0.018$). These results suggest that the development of late immune responses is associated with changes in PSADT.

The evaluation of T-cell immune responses to target self antigens after vaccine clinical trials presents several challenges. Antigen-specific T-cells can be evaluated by their peptide target specificity, proliferative capacity, cytokine secretion, cytolytic activity, and membrane markers of activation. At present, the best measure of antigen-specific T-cells is unknown, as is the optimal time to evaluate immune responses. In our current analysis, we evaluated both humoral responses determined by peptide-specific IgG levels using a Luminex system and antigen-specific CD8+ T-cell responses by using IFN-γ ELISPOT assays, to provide a more direct quantitative assessment after immunization. Delayed 50% PSA decline and prolongation of PSADT were observed in patients with positive IgG and T-cell respkonses, and these immune responses were associated with OS. These results suggest that further immunological analysis at multiple time points might be needed to determine whether T-cell response or the development of late immune responses is associated with clinical responses.

Cancer vaccinations do not always extract good immune and/or clinical responses in vaccinated patients. This study showed that IgG responses and prolongation of PSADT during PPV, along with baseline performance status, CRP, and PSA levels, were well correlated with OS in patients with CRPC treated by PPV. These results suggest that risk stratification based on these factors could be helpful for estimating the OS in patients with CRPC treated by immunotherapy.

Despite these encouraging observations, the current study must be interpreted as hypothesis-generating due to several limitations. This single-arm phase II study without a concurrent control arm did not allow estimation of the potential clinical or immune effects of this treatment. Another potential limitation of this study regarding OS is the lack of treatment data after the treatment phase of the trial. Imbalances due to chance may have occurred in treatments after progression. However, only docetaxel has been shown to affect survival in this population of patients, and only by a few months. The median survival of 18.8 months (95% CI, 14.1 to 24 months) observed in this study surpassed the survival that was observed from docetaxel-based clinical trials in a similar population by TAX-327 (median survival, 19.2 months) and South West Oncology Group 9906 (median survival, 17.5 months) [7,8]. Thus, we think it unlikely that a potential imbalance in post-study treatments could explain the survival results.

## Conclusions

This study showed that PPV in patients with CRPC was active and well tolerated, improving survival with immune responses, delayed PSA responses, and prolongation of PSADT. Further randomized trials are needed to confirm these preliminary results.

### Abbreviations

CR: Complete response; CT: Computed tomography; CRPC: Castration-resistant prostate cancer; CTL: Cytotoxic T lymphocytes; EOCG: Eastern cooperative oncology group; HLA: Human leukocyte antigen; IFN- γ: Interferon-γ; IgG: Immunoglobulin G; OS: Overall survival; PBMC: Peripheral blood mononuclear cells; PPV: Personalized peptide vaccination; PSA: Prostate specific antigen; PSADT: Prostate specific antigen doubling time.

### Competing interests

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

### Authors' contributions

NM conceived of the study, and participated in its design and coordination and drafted the manuscript. KI and AY participated in its design and helped to draft the manuscipt. FM, SS, RO performed the clinical trial and collected the data. SM and TS carried out the immunoassays. All authors read and approved the final manuscript.

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### Author details

[1]Clinical Research Division of the Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan. [2]Departments of Urology, Kurume University School of Medicine, Kurume, Japan. [3]Immunology and Immunotherapy, Kurume University School of Medicine, Kurume, Japan. [4]Cancer Vaccine of the Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, Kurume, Japan.

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# Current status of immunotherapy for the treatment of biliary tract cancer

Ryuji Takahashi,[1] Munehiro Yoshitomi,[1] Shigeru Yutani,[2] Takahisa Shirahama,[1] Masanori Noguchi,[3] Akira Yamada,[3] Kyogo Itoh[2] and Tetsuro Sasada[2,*]

[1]Department of Surgery; Kurume University School of Medicine; Kurume, Japan; [2]Department of Immunology and Immunotherapy; Kurume University School of Medicine; Kurume, Japan; [3]Research Center for Innovative Cancer Therapy; Kurume University; Kurume, Japan

**Biliary tract cancer (BTC) is one of the most aggressive malignancies. Although various promising regimens of chemotherapeutic and/or molecular targeted agents have been developed, further treatment modalities, including immunotherapies, still remain to be established for refractory patients who are unresponsive to or relapse after currently available therapeutic options for BTC. Recently, several clinical trials of immunotherapies, including peptide-based vaccines and dendritic cell (DC)-based vaccines, have been reported with promising results. Here we summarize the data from phase I or phase II clinical trials of immunotherapies for BTC. In particular, we introduce our novel immunotherapeutic approach called personalized peptide vaccine (PPV), in which HLA-matched peptides were selected and administered based on the pre-existing host immunity before vaccination, for the treatment of advanced BTC. Further clinical trials would be recommended to prove clinical benefits of these novel immunotherapeutic approaches. Recently concomitant treatments, such as chemotherapies and immune checkpoint blockade, have been reported to enhance the therapeutic effects of cancer immunotherapies through multiple coordinated immune mechanisms. Additional therapies in combination with immunotherapies could produce synergistic effects in the treatment of advanced BTC.**

**Keywords:** biliary tract cancer, peptide vaccine, dendritic cell vaccine, personalized vaccine, immunotherapy, clinical trial, biomarker

**Abbreviations:** BTC, biliary tract cancer; DC, dendritic cell; PPV, personalized peptide vaccine; GEM, gemcitabine; TAA, tumor-associated antigen; WT1, Wilms tumor gene 1; MUC1, mucin 1; SD, stable disease; PD, progressive disease; OS, overall survival; PFS, progression-free survival; HR, hazard ratio; CI, confidence interval

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*Correspondence to: Tetsuro Sasada;
Email: tsasada@med.kurume-u.ac.jp

## Introduction

Biliary tract cancer (BTC) is one of the most aggressive malignancies.[1,2] Only 10% of newly diagnosed patients present with early-stage disease and can be treated by a potentially radical excision of tumors. However, the remaining patients with unresectable, locally advanced and/or metastatic tumors show a poor prognosis, with a median survival of less than one year.[1,2] For advanced or recurrent BTC that are ineligible for surgery, various promising regimens of chemotherapeutic and/or molecular targeted agents have been studied.[1-4] For example, a combination of chemotherapeutic agents, gemcitabine (GEM) and cisplatin, has recently demonstrated a promising result in a randomized phase III trial in advanced BTC patients.[3] However, further treatment modalities still remain to be established for refractory patients who are unresponsive to or relapse after currently available therapeutic regimens for BTC.

Infiltration of different subsets of immune cells, including lymphocytes, macrophages, DCs and granulocytes, as well as immune-related microenvironments have been demonstrated to foster or inhibit tumor progression and/or metastatic potential in various types of cancers.[5,6] In BTC, higher frequencies of tumor-infiltrating $CD8^+$ cytotoxic T cells and/or $CD4^+$ T cells have been shown to be closely associated with favorable patient prognosis.[7,8] These findings have provided the rationale

**Table 1.** List of clinical trials of immunotherapies for biliary tract cancer

| Type of vaccine | Disease condition | Phase of trial | Combined treatment | No. of patient | Clinical response | Median OS | Grade 3/4 toxicities (%) | Humoral response (%) | Cellular response (%) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| MUC1 peptide | Advanced | I | (-) | 3 | PD 100% | NA | 0 | 0 | 0 | 20 |
| MUC1 peptide-loaded DCs | Adjuvant | I | (-) | 2 | No recurrence, 50% | NA | 0 | NA | NA | 21 |
| WT1 peptide | Advanced | I | GEM | 16 | SD 50%, PD 50% | 288 d | 0 | NA | 56 | 22 |
| Tumor lysate-pulsed DCs plus activated T cell transfer | Adjuvant | I | (-) | 36 | PFS; 18.3M (vs 7.7M) | 31.9M (vs 17.4M) | NA | NA | NA | 24 |
| Personalized peptide vaccine (PPV) | Advanced–(chemo-resistant) | II | chemotherapy | 25 | SD 80%, PD 20% | 207 d | 0 | 35 | 47 | 32 |

DCs, dendritic cells; GEM, Gemcitabine; OS, overall survival; PFS, progression-free survival; SD, stable disease; PD, progressive disease; M, months; NA, not available.

for further development of immunotherapies as a novel treatment modality against BTC. Here we summarize the current status of immunotherapies against BTC.

## Recent Developments of Immunotherapeutic Approaches Against BTC

The field of cancer immunotherapy has drastically moved forward during these two decades since the first discovery of a tumor-associated antigen (TAA) recognized by cytotoxic T lymphocytes in 1991.[9-12] Advancement of molecular biological and immunological techniques has helped identify a large number of TAAs and peptide epitopes applicable as cancer immunotherapies.[13] For example, BTC has been reported to express a variety of TAAs, such as Wilms tumor gene 1 (WT1),[14] mucin 1 (MUC1)[15-17] and mutated K-RAS,[18,19] as potential targets for immunotherapies. Several clinical trials of immunotherapies targeting these molecules have recently been reported with promising results (**Table 1**).

Two groups employed a 100-mer peptide derived from MUC1 for the vaccination to BTC patients.[20,21] Yamamoto et al. reported a phase I clinical trial of vaccination with a 100-mer peptide consisting of the extracellular tandem repeat domain of MUC1 and incomplete Freund's adjuvant (Montanide ISA51) in patients with advanced pancreatic cancer (n = 6) or BTC (n = 3).[20] This study showed the safety of this vaccine formulation, but produced no substantial effects on antigen-specific immunological parameters or clinical outcomes in the vaccinated BTC patients. Lepisto et al. performed a Phase I/II clinical trial of vaccination with autologous DCs loaded with the 100-mer MUC1 peptide as an adjuvant therapy against pancreatic cancer (n = 10) or BTC (n = 2) patients following resection of their primary tumors.[21] The vaccine was well tolerated and no toxicity was observed. One of two patients with stage II intrahepatic cholangiocarcinoma had a long survival time without recurrence, although this patient showed no induction or boosting of MUC1 specific immune responses after vaccination.

Kaida et al. conducted an open-labeled, dose-escalation phase I trial of WT1 peptide vaccine combined with GEM to evaluate the safety and optimal immunological dose of this vaccine in HLA-A*0201, -A*0206, and/or -A*2402 positive patients with advanced pancreatic cancer (n = 9) or BTC (gallbladder carcinomas, n = 8; intrahepatic cholangiocarcinomas; n = 4; and extrahepatic cholangiocarcinomas, n = 4).[22] In this trial, 6 doses of GEM and 4 doses of WT1 peptide (1 or 3 mg) emulsified in incomplete Freund's adjuvant (Montanide ISA51) were administered. The adverse events were comparable to those with GEM alone, confirming the safety of this combination therapy. WT1-specific T cells in peptide-stimulated culture were detected by tetramer assay in 56% (9 of 16) of BTC patients. The clinical responses at 2 mo after vaccination showed 8 stable diseases (SD) and 8 progressive diseases (PD), and the median overall survival (OS) time for BTC was 288 d. Based on these promising data, the same group has started a phase I and randomized phase II study with WT1 peptide vaccine in combination with GEM and cisplatin for chemo-naive patients with unresectable or recurrent BTC.[23]

Shimizu et al. reported a phase I trial of autologous tumor lysate-pulsed DCs in combination with ex vivo CD3-activated T-cell transfer in an adjuvant setting for 36 postoperative patients with intrahepatic cholangiocarcinomas.[24] The median progression-free survival (PFS) and OS time of the patients receiving this adjuvant immunotherapy were 18.3 and 31.9 mo, respectively, which were significantly better than those of the control group receiving surgery alone [7.7 mo (p = 0.005) and 17.4 mo (0.022),

respectively]. In particular, patients with skin reactions (> 3 cm) at the vaccine site showed dramatically better prognosis. These results suggested a potential clinical benefit of this therapy for preventing recurrence and achieving long-term survival in intrahepatic cholangiocarcinoma patients, although a randomized trial will be needed for its confirmation.

## Personalized Peptide Vaccine for BTC Patients

The anti-tumor immunity might differ widely among individual cancer patients, since the tumor cell characteristics and the host immune cell repertoires are reported to be quite diverse and heterogeneous among patients, even among those with identical HLA types and the same pathological types of cancer.[25-28] 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 HLA-matched vaccine peptides are selected for vaccination based on the pre-existing host immunity from a list of vaccine candidates.[29,30] We have 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.[31]

Recently, we conducted a phase II clinical trial of PPV for 25 chemo-resistant BTC patients (gallbladder carcinomas, n = 7; extrahepatic cholangiocarcinomas, n = 11; intrahepatic cholangiocarcinomas, n = 6; and periampullary carcinoma, n = 1) to evaluate the feasibility of this treatment and to identify potential biomarkers.[32] A maximum of 4 peptides were selected in consideration of the pre-existing host immunity before vaccination, as assessed by the titers of IgGs specific to each of the 31 different vaccine candidates [12 peptides for HLA-A2, 16 peptides for HLA-A24, 9 peptides for HLA-A3 supertypes (-A3, -A11, -A31, and -A33), and 4 peptides for HLA-A26], whose safety and immunological effects for other types of cancers were confirmed in previously conducted clinical studies. The selected peptides (3 mg/each peptide) were emulsified in incomplete Freund's adjuvant (Montanide ISA51) and subcutaneously administrated (weekly for 6 consecutive weeks and then bi-weekly thereafter) in combination with chemotherapeutic agents without severe adverse events. The median OS time was 207 d. In 10 patients who were radiologically evaluated before and after vaccination, the clinical response was classified as SD in 8 patients and PD in 2 patients. Humoral and T cell responses specific to the vaccine antigens were substantially induced in a subset of the vaccinated patients (35% and 47%, respectively). In the multivariate Cox regression analysis, lower IL-6 levels, higher albumin levels, and greater numbers of selected vaccine peptides were significantly favorable factors for OS [hazard ratio (HR) = 1.123, 95% confidence interval (CI) = 1.008 - 1.252, p = 0.035; HR = 0.158, 95% CI = 0.029 - 0.860, p = 0.033; HR = 0.258, 95% CI = 0.098 - 0.682, p = 0.006; respectively], suggesting that the evaluation of inflammation, nutritional status, and pre-existing antigen-specific immunity before vaccination could be useful for selecting appropriate BTC patients who would benefit from PPV. Based on this finding, we are planning an early phase clinical trial to reveal whether or not the blockade 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 BTC patients who show higher levels of plasma IL-6.[33,34]

## Conclusions

Several clinical trials of immunotherapies for BTC have been reported with promising immunological responses and/or clinical outcomes. Further randomized trials would be essential to prove clinical benefits of these novel immunotherapies. Recently concomitant treatments, such as chemotherapies and immune checkpoint blockade, have been reported to enhance the therapeutic effects of cancer immunotherapies through multiple coordinated immune mechanisms, including activation of antigen-presenting cells or cytotoxic T cells and removal of suppressor cells.[35,36] Additional therapies in combination with immunotherapies could produce synergistic effects in the treatment of advanced BTC.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.


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