Survival analysis of multiple peptide vaccination for the selection of correlated peptides in urological cancers

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Peptide-based cancer vaccines are able to induce strong immune responses, but their clinical results are unsatisfactory. To determine clinically correlated peptides, we analyzed survival data from urological cancer patients treated by personalized peptide vaccination (PPV), in which different multiple peptides were used for individual patients based on human leukocyte antigen (HLA) type and pre-existing immunity. Survival data were obtained from a database of 265 urological cancer patients treated in 5 clinical PPV trials comprising 154 patients with castration-resistant prostate cancer (CRPC) and 111 patients with advanced urothelial cancer (UC). Expression of tumor-associated antigens (TAA) was evaluated in 10 prostate cancer tissues, 4 metastatic lymph nodes from prostate cancer, and 10 UC tissues using immunohistochemical staining. Clinical efficacy of individual peptides for overall survival was evaluated by the Cox proportional hazards regression model. All TAA coding candidate peptides used in PPV treatment were expressed in tumor cells from prostate cancer and UC samples except for p56Lck in both, and prostate-specific antigen (PSA), prostatic acid phosphatase (PAP) and prostate-specific membrane antigen (PSMA) in the UC samples. Patients with the following peptides had a significantly longer survival than patients without the peptides (hazard ratio <1.0, 95% confidence intervals <1.0 and P < .05): SART3-109, PTHrP-102, HNPRL-140, SART3-302 and Lck-90 in CRPC patients, and EGF-R-800, Lck-486, PSMA-624, CypB-129 and SART3-734 in advanced UC patients, respectively. Correlated peptides selected using both survival data and pre-existing immunity for PPV treatment may enhance the clinical benefits for urological cancer patients.

KEYWORDS
immunotherapy, peptide-based cancer vaccine, prostate cancer, survival analysis, urothelial cancer

Abbreviations: CI, confidence interval; CP, correlated peptide; CRPC, castration-resistant prostate cancer; HLA, human leukocyte antigen; HR, hazard ratio; IFN-γ, interferon-γ; IHC, immunohistochemical staining; OS, overall survival; PAP, prostatic acid phosphatase; PPV, personalized peptide vaccine; PS, performance status; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; TAA, tumor-associated antigen; TIL, tumor-infiltrating lymphocyte; UC, urothelial cancer.

Clinical Trials Registry: UMIN000001850, UMIN000005329, UMIN000010290, UMIN000001854 and UMIN000003157.

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1 | INTRODUCTION

Peptide-based cancer vaccines are considered a promising therapeutic strategy; however, their clinical results have been unsatisfactory for the past 2 decades.1-3 One reason for this is the difficulty in selecting efficient TAA because of the heterogeneity of tumor cells and the diversity of immune responses. After much consideration, PPV was developed in which individuality of tumor cells and the diversity of immune responses. After much consideration, PPV was developed in which individual

2 | PATIENTS AND METHODS

2.1 | Candidate peptides

In PPV treatment, 31 candidate peptides from 15 TAA were prepared under the conditions of Good Manufacturing Practice using a Multiple Peptide System (San Diego, CA, USA). All 31 candidate peptides were CTL epitopes restricted to the HLA-A2, -A24 or -A3 families (HLA-A3, -A11, -A31 or -A33) of MHC class I molecules, which covered the majority of the general population. Names of peptides, coding TAA, amino acid sequences, and HLA types are given in Table 1. Expression levels of the 15 TAA that code the peptides used for PPV treatment were examined by IHC of primary prostate cancer tissues (n = 10), metastatic lymph nodes from prostate cancer (n = 10), and primary UC tissues (n = 10), obtained from nonvaccinated patients. Detailed methods, including antibodies used for staining, were previously described.7,9-17

2.2 | Patient selection

From April 2009 to April 2013, the Cancer Vaccine Center of Kurume University in Japan conducted 5 prospective phase II clinical trials of PPV treatment for advanced urological cancers involving 154 patients with CRPC (UMIN Clinical Trials Registry: UMIN000001850, UMIN000005329 and UMIN000010290) and 111 patients with advanced UC progressing after platinum-containing chemotherapy (UMIN Clinical Trials Registry: UMIN000001854 and UMIN00003157). Characteristics of the CRPC patients from 3 trials and UC patients from 2 trials were similar. All patients provided written informed consent for study participation and data collection. The results of all 5 clinical trials were reported previously.6,18-21 In the present study, the data of these trials were used to analyze the

| Table 1 | Candidate peptides used in the present study |
|-----------------------------------------|-----------------------------------------------|
| Peptide name | Source | Position of peptide | Amino acid sequence | HLA type |
|-----------------|--------|---------------------|---------------------|----------|
| SART2-93        | SART2  | 93-101              | DYSARWNEI          | A24      |
| SART3-109       | SART3  | 109-118             | VYDYNCHVDL         | A24,A3 family* |
| Lck-208         | p56 lck | 208-216             | HYTNASDGL          | A24      |
| PAP-213         | PAP    | 213-221             | LYESVHNF           | A24      |
| PSA-248         | PSA    | 248-257             | HYRKWIKDTI         | A24      |
| EGF-R-800       | EGF-R  | 800-809             | DYVREHKDNI         | A24      |
| MRP3-503        | MRP3   | 503-511             | LYWEPSFL           | 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             | TYSVSFDGL          | A24      |
| EZH2-735        | EZH2   | 735-743             | KYGIEREM           | A24      |
| PTHR-P-102      | PTHR-P | 102-111             | RYLTQETNKV         | A24      |
| CypB-129        | Cyclophilin B | 129-138          | KLKHYGPQWW         | A2,A3 family* |
| Lck-246         | p56 lck | 246-254             | KLVERLGA           | A2       |
| Lck-422         | p56 lck | 422-430             | DVWSFGILL          | A2,A3 family* |
| MAP-432         | pMPAkk | 432-440             | DLLSHAFFA          | A2       |
| WHSC2-103       | WHSC2  | 103-111             | ASLSDPWV           | A2,A3 family* |
| HNRPL-501       | HNRPL  | 501-510             | NVLHFFNAPL         | A2       |
| UBE-43          | UBE2V  | 43-51               | RLQEWCSV1          | A2       |
| UBE-85          | UBE2V  | 85-93               | LIADFLSGL          | A2       |
| WHSC2-141       | WHSC2  | 141-149             | ILGELREKV          | A2       |
| HNRPL-140       | HNRPL  | 140-148             | ALVEFEDVL          | A2       |
| SART3-302       | SART3  | 302-310             | LQLAEPAPR          | A2       |
| SART3-309       | SART3  | 309-317             | RCAEQYAI           | A2       |
| SART3-511       | SART3  | 511-519             | WLEYYNLER          | A3 family* |
| SART3-734       | SART3  | 734-742             | QIRPFSNR           | A3 family* |
| Lck-90          | p56 lck | 90-99               | ILEQSGEWKK         | A3 family* |
| Lck-449         | p56 lck | 449-458             | VIQNLERGYR         | A3 family* |
| PAP-248         | PAP    | 248-257             | GIHKQKEKSR         | A3 family* |

*A3 family, HLA-A3, A11, A31 and A33. HLA, human leukocyte antigen; TAA, tumor-associated antigen.
peptides that correlated with clinical benefits. Common inclusion or exclusion criteria for the 5 clinical trials of PPV treatment were as follows: eligible patients were aged ≥18 years and had ECOG PS of 0 or 1, life expectancy of at least 12 weeks, positive HLA-A2, -A24 or -A3 family (-A3, -A11, -A31 and -A33) status, positive IgG responses to at least 2 of the 31 different candidate peptides, and adequate bone marrow function, hepatic function and renal function. Exclusion criteria included acute infection, history of severe allergic reactions, pulmonary, cardiac or other systemic diseases, or other inappropriate conditions for enrollment as judged by clinicians. The procedure for PPV treatment was as follows. Each of the selected peptides based on HLA type and level of IgG titer was mixed with incomplete Freund’s adjuvant (Montanide ISA-51VG; Seppic, Paris, France), and a 1.5-mL emulsion (3 mg/peptide) of a maximum of 4 peptides was injected s.c. into the lateral thigh area once a week for 6 or 8 weeks as the first cycle of PPV treatment. After the first cycle, PPV dosage was carried out in 2, 3- or 4-week intervals until withdrawal of consent or unacceptable toxicity.

2.3 Estimation of clinical efficacy of individual peptides

According to HLA type, survival data for each patient were divided into 2 groups, with or without individual peptides, using individual peptides at the first cycle. Cox proportional hazards regression analyses were then carried out to calculate HR, 95% CI, and P-values for the treatment effects of individual peptides in relation to OS, and to select peptides that correlated with OS. Peptides used for only a few patients (<10%) were excluded from the Cox proportional hazards regression analyses to avoid bias.

2.4 Immune responses to individual peptides

Individual peptide-specific IgG and CTL responses were analyzed in blood samples before and after the first cycle of PPV treatment. IgG responses were measured by the Luminex system (Luminex, Austin, TX, USA) using plasma, and the CTL responses were evaluated by IFN-γ ELISPOT assay (MBL, Nagoya, Japan) using PBMC as previously described. Higher IgG titers to vaccinated peptides at the end of the first cycle than those in prevaccination plasma and more than 30 spots for the corresponding peptide in PBMC as observed by IFN-γ ELISPOT assay were considered to be positive immune responses.

2.5 Statistics

Baseline characteristics, vaccinated peptides, immunological responses to vaccinated peptides, OS during PPV treatment, and follow-up were extracted from the database of the 5 clinical PPV trials. Student’s t test and the chi-squared test were used to compare quantitative and categorical variables, respectively. OS was calculated as the time in months from the date of study enrollment to death or to the date of last contact. Time-to-event endpoints were analyzed using the Kaplan-Meier method, and between-group comparisons for OS were conducted using the log-rank test. Clinical efficacy of individual peptides for OS was evaluated by univariate and multivariate analyses with the Cox proportional hazards regression model, and HR and 95% CI were calculated. Favorable peptides for clinical efficacy (HR <1.0) in the univariate analysis were included in the multivariate analysis. All reported P-values were 2-sided, and P-values <.05 were considered significant. JMP version 12 or SAS version 9.4 software (SAS Institute Inc., Cary, NC, USA) was used to carry out all analyses.

3 RESULTS

3.1 Expression of tumor antigens

In the 10 primary prostate cancer tissues tested, 4 TAA (PSA, PSMA, PTHrP, and CypB) were highly expressed in the majority (>80%) of tumor cells and nonmalignant cells, whereas 6 TAA (PAP, UBE2V, HNRPL, SART2, SART3, and ppMAPkkk) were modestly expressed in the majority (>80%) of tumor cells and nonmalignant cells. WHSC2 and epidermal growth factor receptor (EGFR) were expressed in half of the tumor cells and nonmalignant cells from 5 samples. There were no TAA expressed only in nonmalignant cells. Although p56Lck was not expressed in any tumor cells tested, it was expressed in TIL from all samples. EZH2 and MRP3 were not expressed in any tumor cells or nonmalignant cells from the 10 samples tested. Four metastatic lymph nodes from advanced prostate cancer patients were also provided for the study and showed similar results as above with the exception of EZH2 and MRP3, which became detectable in 3 and 1 of the 4 samples tested, respectively. Representative results of IHC in prostate cancer tissues are shown in Figure 1A.

In the 10 primary UC tissues tested, 5 TAA (SART2, SART3, EZH2, ppMAPkkk, and WHSC2) were highly expressed in the majority of both tumor cells and nonmalignant cells, whereas 4 TAA (UBE2V, HNRPL, PTHrP, and EGFR) were modestly expressed in the majority of both tumor cells and normal cells. CypB and MRP3 were expressed in both tumor cells and nonmalignant cells from 6 and 3 of 10 samples tested, respectively. Similar to the results of IHC for prostate cancer tissues, p56Lck was not expressed in any of the tumor cells tested but was expressed in TIL from all samples. PSA, PAP, and PSMA were not expressed in any urothelial cancer cells or normal cells from the 10 samples tested. Representative results of IHC in urothelial cancer tissues are shown in Figure 1B.

3.2 Baseline characteristics

Table 2 shows the baseline characteristics of the advanced urological cancer patients analyzed in this study classified by HLA type. There was no significant difference in age, PS, PSA levels, Gleason score, metastatic site or prior chemotherapy in CRPC patients among HLA types, or in age, PS, primary tumor site, metastatic site or prior chemotherapy in advanced UC patients among HLA types. All patients were refractory to the first-line treatment or chemotherapy.
3.3 Estimation of correlated peptides

Peptides used in <10% of all patients were excluded from the univariate Cox regression analysis to avoid bias as follows: Lck208, MRP3-503, and EZH2-735 for HLA-A24, Lck-422, and UBE2V-85 for HLA-A2, and Lck-422 for the HLA-A3 family in CRPC patients (Table 3); MRP3-503, SART2-161, and EZH2-735 for HLA-A24, Lck-422, and UBE2V-85 for HLA-A2, and Lck-422 for the HLA-A3 family in advanced UC patients (Table 4). The remaining peptides with HR <1.0 were then used for multivariate Cox regression analysis to calculate the HR, 95% CI and P-values. In CRPC patients, SART3-109 (HR 0.55, 95% CI 0.31-0.94; P = .03) and PTHrP-102 (HR 0.45, 95% CI 0.18-0.96; P = .04) for HLA-A24, HNPRL-140 (HR 0.14, 95% CI 0.01-0.78; P = .02), and SART3-302 (HR 0.39, 95% CI 0.15-0.97; P = .04) for HLA-A2, and Lck-90 (HR 0.21, 95% CI 0.03-0.94; P = .04) for the HLA-A3 family were considered to be correlated peptides based on significant differences with unused peptides (Table 3). In advanced UC patients, EGF-R-800 (HR 0.35, 95% CI 0.15-0.73; P = .005), Lck-486 (HR 0.54, 95% CI 0.3-0.97; P = .04) and PSMA-624 (HR 0.32, 95% CI 0.1-0.95; P = .04) for HLA-A24, CypB-129 (HR 0.17, 95% CI 0.03-0.67; P = .01) for HLA-A2, and SART3-734 for the HLA-A3 family were also considered to be correlated peptides (Table 4). All correlated peptides showed peptide-specific IgG and CTL responses at the end of the first cycle of PPV treatment (Table 5).

3.4 Effectiveness of peptides correlated with OS

We also carried out an OS subgroup analysis based on groups stratified by use of correlated peptides according to HLA type (Figure 2). In CRPC, median OS times for patients with correlated peptides were significantly longer than for those without correlated peptides: 31.5 vs 16.9 months for HLA-A24 (P = .01), 30.6 vs 8.7 months for HLA-A2 (P < .001) and 27.5 vs 18.1 months for HLA-A3 family (P = .02). The median OS times for advanced UC patients with
correlated peptides were also significantly longer than for those without correlated peptides: 8.3 vs 4.5 months for HLA-A24 \((P = .006)\), 13.4 vs 7.2 months for HLA-A2 \((P = .02)\) and 29.9 vs 9.7 months for HLA-A3 family \((P = .01)\).

**DISCUSSION**

In the present study, we indicated using 265 advanced urological cancer patients treated by PPV that correlated peptides selected by both

| TABLE 2 Baseline characteristics of advanced urological cancer patients |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Factor          | CRPC (n = 154)   | Advanced UC (n = 111) |
|                 | HLA type        | HLA type        |
|                 | A24 (n = 102)   | A2 (n = 73)     | A3 family (n = 17) | A2 (n = 22)     | A3 family (n = 16) |
|                 | A2 (n = 35)     | A2 (n = 35)     | A3 family (n = 17) | A2 (n = 22)     | A3 family (n = 16) |
| Age, y          | Median 68       | 65              | 70              | 66.5            | 63.5            | .81             |
|                 | Range 43-85     | 42-83           | 59-84           | 40-80           | 45-78           |
| Gender          | Male 102        | 53 (73%)        | 35              | 17              | 19 (26%)        | .79             |
|                 | Female —        | 17 (77%)        | 17              | 13 (81%)        | 5 (23%)         |                 |
|                 | —               | 13 (81%)        | 3 (19%)         |                 |                 |                 |
| ECOG PS         | 0 83 (81%)      | 59 (81%)        | 26 (74%)        | 15 (88%)        | .46             |
|                 | 1 19 (19%)      | 14 (19%)        | 9 (26%)         | 2 (12%)         |                 |
|                 | —               | 15 (68%)        | 2 (12%)         | 7 (32%)         | 3 (19%)         |
| PSA, ng/mL      | Median 38.5     | 50 (100%)       | 17.9            | 0.02-741        | 20.2            | .31             |
|                 | Range 0-10,895  | 0-10,895        | 0.02-741        | 1.4-278         | —               |                 |
| Gleason score   | <8 22 (22%)     | 73 (100%)       | 11 (31%)        | 3 (18%)         | .41             |
|                 | >8 80 (78%)     | 22 (30%)        | 24 (69%)        | 14 (82%)        |                 |
| Primary tumor sites | Prostate 102  | 6 (5%)          | 35              | 17              | —               | .07             |
|                 | Bladder —       | 30 (45%)        | 35              | 17              | —               |                 |
|                 | Ureter or pelvis— | 30 (45%)        | 35              | 17              | —               |                 |
| Metastatic sites | None 7 (7%)     | 6 (8%)          | 2 (6%)          | 1 (6%)          | .08             |
|                 | Lymph node only 12 (12%) | 22 (30%)        | 6 (8%)          | 1 (5%)          | 1 (5.5%)        |
|                 | Bone only 41 (40%) | 11 (31%)        | 22 (30%)        | 7 (32%)         | 2 (13%)         |
|                 | Lymph node with bone 32 (31%) | 11 (31%)        | 12 (30%)        | 7 (32%)         | 2 (13%)         |
|                 | Lung only 0      | 15 (21%)        | 1 (1%)          | 1 (5%)          | 1 (5.5%)        |
|                 | Lymph node with lung 1 (1%) | 15 (21%)        | 1 (1%)          | 1 (5%)          | 1 (5.5%)        |
|                 | Others with liver 4 (4%) | 4 (5%)          | 1 (1%)          | 1 (5%)          | 1 (5.5%)        |
|                 | Others without liver 6 (6%) | 4 (5%)          | 1 (1%)          | 1 (5%)          | 1 (5.5%)        |
| Prior radical surgery | 17 (17%) | 43 (59%)        | 1 (3%)          | 1 (6%)          | .07             |
|                 | Prior irradiation 40 (39%) | 6 (17%)        | 6 (35%)         | 3 (13%)         | 0.06             |
|                 | Prior docetaxel 57 (56%) | 18 (51%)        | 6 (35%)         | 3 (13%)         | 0.29             |
|                 | Prior GC or MVAC — | 73 (100%)       | 22 (30%)        | 16 (100%)       |                 |
| Vaccination, times | Median 18       | 10              | 17              | 8               | 11.5            |
|                 | Range 2-68      | 1-44            | 1-48            | 1-2.9           | 2-38            |
| Follow up, months | Median 20.5    | 5.5             | 16.2            | 6.3             | 10.5            |
|                 | Range 0.6-87.4 | 0.4-54          | 2.5-40.4        | 0.6-40.7        | 1.72-6.9        |

CRPC, castration-resistant prostate cancer; GC, gemcitabine and cisplatin; HLA, human leukocyte antigen; MVAC, methotrexate, vinblastine, doxorubicin, and cisplatin; PS, performance status; PSA, prostate-specific antigen; UC, urothelial cancer; —, not applicable.
immunogenicity of TAA, inadequate peptide selection and tumor clinical benefits. This may be due to many factors, including the poor were able to induce peptide

Importantly, analyzed survival data were obtained from a cohort of uro-

TABLE 3 Univariate and multivariate analyses for contributing peptides in CRPC patients

| HLA          | Peptides   | No. of pts with/without | Univariate |         |         |         |         |         |
|--------------|------------|-------------------------|------------|---------|---------|---------|---------|---------|
| A24 (n = 102)| SART2-93   | 74/28                   | 1.14       | 0.71    | 1.9     | .60     |         |         |
|              | SART3-109  | 21/81                   | 0.58       | 0.32    | 0.98    | .04     |         |         |
|              | Lck-208    | 8/94                    | —          | —       | —       | —       | —       | —       |
|              | PAP-213    | 30/72                   | 1.12       | 0.68    | 1.79    | .64     |         |         |
|              | PSA-248    | 51/51                   | 1.20       | 0.77    | 1.87    | .41     |         |         |
|              | EGF-R-800  | 24/78                   | 1.22       | 0.72    | 1.96    | .45     |         |         |
|              | MRP3-503   | 7/95                    | —          | —       | —       | —       | —       | —       |
|              | MRP3-1293  | 15/87                   | 1.05       | 0.53    | 1.91    | .87     |         |         |
|              | SART2-161  | 12/90                   | 0.94       | 0.45    | 1.76    | .85     |         |         |
|              | Lck-486    | 56/46                   | 1.47       | 0.94    | 2.32    | .09     |         |         |
|              | Lck-488    | 64/38                   | 1.09       | 0.71    | 1.73    | .70     |         |         |
|              | PSMA-624   | 10/92                   | 1.23       | 0.55    | 2.41    | .59     |         |         |
|              | EZH2-735   | 4/98                    | —          | —       | —       | —       | —       | —       |
|              | PThrP-102  | 11/91                   | 0.48       | 0.19    | 1.01    | .05     |         |         |
| A2 (n = 35)  | CypB-129   | 15/20                   | 1.07       | 0.47    | 2.41    | .87     |         |         |
|              | Lck-246    | 20/15                   | 0.76       | 0.34    | 1.7     | .50     | 0.59    | 0.22   |
|              | Lck-422    | 0/35                    | —          | —       | —       | —       | —       | —       |
|              | ppMAPkkk-432 | 11/24              | 1.59       | 0.64    | 3.64    | .30     |         |         |
|              | WHSC2-103  | 15/20                   | 1.17       | 0.52    | 2.64    | .70     |         |         |
|              | HNRPL-501  | 9/26                    | 1.42       | 0.55    | 3.3     | .45     |         |         |
|              | UBE2V-43   | 15/20                   | 0.86       | 0.37    | 1.92    | .71     | 1.28    | 0.47   |
|              | UBE2V-85   | 3/32                    | —          | —       | —       | —       | —       | —       |
|              | WHSC2-141  | 10/25                   | 1.11       | 0.43    | 2.58    | .82     |         |         |
|              | HNRPL-140  | 4/31                    | 0.16       | 0.009   | 0.79    | .02     | 0.07    | 0.003  |
|              | SART3-302  | 14/21                   | 0.73       | 0.31    | 1.65    | .45     | 0.39    | 0.148  |
|              | SART3-309  | 12/23                   | 0.98       | 0.42    | 2.23    | .96     | 0.64    | 0.23   |
| A3 family (n = 17)| SART3-109 | 3/14                   | 0.67       | 0.21    | 2.66    | .60     | 1.19    | 0.09   |
|              | SART3-511  | 8/9                     | 0.36       | 0.08    | 1.25    | .11     | 0.60    | 0.07   |
|              | SART3-734  | 12/5                    | 0.99       | 0.31    | 3.75    | .98     | 1.03    | 0.21   |
|              | Lck-90     | 8/9                     | 0.18       | 0.03    | 0.72    | .01     | 0.21    | 0.028  |
|              | Lck-449    | 7/10                    | 2.22       | 0.66    | 8.57    | .20     | —       | —       |
|              | PAP-248    | 3/14                    | 0.26       | 0.01    | 1.39    | .13     | 0.31    | 0.01   |
|              | Lck-422    | 1/16                    | —          | —       | —       | —       | —       | —       |
|              | CypB-129   | 9/8                     | 4.81       | 1.12    | 33.13   | .03     | —       | —       |
|              | WHSC2-103  | 6/11                    | 1.04       | 0.27    | 3.51    | .95     | —       | —       |

Peptides used for <10% of patients were excluded from the univariate analysis.
Correlated peptides are shown in italics and significant P-values are in shown in bold text. CI, confidence interval; CRPC, castration-resistant prostate cancer; HR, hazard ratio; —, not applicable.

pre-existing immunity and efficacy for OS significantly increased survival compared with peptides selected only by pre-existing immunity. Importantly, analyzed survival data were obtained from a cohort of urological cancer patients who had equally poor prognostic backgrounds.

Most peptides previously used in cancer vaccines in clinical trials were able to induce peptide-specific IgG or CTL responses but had no clinical benefits. This may be due to many factors, including the poor immunogenicity of TAA, inadequate peptide selection and tumor heterogeneity. In addition, the limited clinical efficacy may be a result of not knowing the immunological status of patients, which leads to mismatches between used peptides and heterogeneous immune cell repertoires. Upregulation of immune checkpoint molecules in tumor tissues after immunotherapy may also be a mechanism of peptide-based vaccine failure. IFN-γ secreted by activated T cells mediates programmed cell death 1 (PD-1) expression by TIL and programmed cell death ligand 1 (PD-L1) expression by tumor cells,
respectively. Combination therapy with cancer vaccination and anti-PD-1 antibody in murine cancer models enhanced TIL antigen reactivity and prolonged the survival of tumor-bearing mice. We therefore expect combination therapy with peptide-based vaccination and immune checkpoint blockade to be an effective strategy for peptide-based vaccine failure or immune checkpoint blockade failure.

Unlike other peptide-based vaccines, PPV is a novel immunotherapy approach for cancer patients. In PPV treatment, many suitable candidate peptides are selected based on HLA type and the pre-existing host immunity. Conventional peptide-based vaccines containing only 1 peptide may not initiate a specific antitumor response in tumor cell variants because of the loss or reduction in TAA. In this study, we found that the majority of TAA coding candidate peptides used in PPV treatment were expressed in tumor cells from prostate cancer or UC samples, except for p56Lck in both, and PSA, PAP and PSMA in the UC samples. However, p56Lck was expressed in TIL from all

### TABLE 4 Univariate and multivariate analyses for contributing peptides in advanced UC patients

| HLA   | Peptides     | No. of pts with/without | Univariate HR, 95% CI, P-value | Multivariate HR, 95% CI, P-value |
|-------|--------------|-------------------------|---------------------------------|----------------------------------|
| A24 (n = 73) | SART2-93     | 51/22                   | 0.86 (0.51-1.52) .59            | 0.61 (0.32-1.23) .16            |
|       | SART3-109    | 10/63                   | 1.31 (0.63-2.49) .45            | —                               |
|       | Lck-208      | 14/59                   | 0.83 (0.39-1.57) .57            | 0.71 (0.3-1.57) .41            |
|       | PAP-213      | 15/58                   | 0.64 (0.32-1.18) .16            | 0.52 (0.25-1.02) .06            |
|       | PSA-248      | 23/50                   | 0.97 (0.55-1.63) .90            | 1.05 (0.59-1.83) .87            |
|       | EGF-R-800    | 12/61                   | 0.55 (0.26-1.04) .07            | 0.35 (0.15-0.73) .005           |
|       | MRP3-503     | 3/70                    | —                               | —                               |
|       | MRP3-1293    | 11/62                   | 0.75 (0.35-1.46) .42            | —                               |
|       | SART2-161    | 6/67                    | —                               | —                               |
|       | Lck-486      | 32/41                   | 0.85 (0.5-1.41) .53            | 0.54 (0.3-0.97) .04            |
|       | Lck-488      | 52/21                   | 1.16 (0.67-2.08) .61            | —                               |
|       | PSMA-624     | 8/65                    | 0.69 (0.24-1.57) .40            | 0.32 (0.1-0.95) .04            |
|       | EZH2-735     | 2/71                    | —                               | —                               |
|       | PTHrP-102    | 11/62                   | 1.33 (0.61-2.57) .45            | —                               |
| A2 (n = 22) | CypB-129     | 9/13                    | 0.27 (0.08-0.81) .02            | 0.17 (0.03-0.67) .01           |
|       | Lck-246      | 7/15                    | 0.58 (0.19-1.61) .30            | 0.33 (0.07-1.24) .1             |
|       | Lck-422      | 1/21                    | —                               | —                               |
|       | ppMAPkkk-432 | 4/18                    | 1.92 (0.42-6.48) .36            | —                               |
|       | WHSC2-103    | 7/15                    | 0.51 (0.16-1.43) .20            | —                               |
|       | HNRPL-501    | 3/19                    | 1.00 (0.22-3.23) 1.00           | 1.15 (0.14-10.59) .9            |
|       | UBE2V-43     | 11/11                   | 0.79 (0.27-2.16) .66            | 0.68 (0.17-2.79) .58           |
|       | UBE2V-85     | 1/21                    | —                               | —                               |
|       | WHSC2-141    | 12/10                   | 0.82 (0.3-2.32) .69            | 0.50 (0.13-1.96) .31           |
|       | HNRPL-140    | 5/17                    | 3.97 (0.99-14.19) .05           | —                               |
|       | SART3-302    | 9/13                    | 1.00 (0.31-2.79) 1.00           | —                               |
| A3 family (n = 16) | SART3-109 | 3/13                    | 2.98 (0.64-10.85) .15           | —                               |
|       | SART3-511    | 8/8                     | 1.09 (0.32-3.45) .89            | —                               |
|       | SART3-734    | 10/6                    | 0.22 (0.06-0.8) .02            | 0.22 (0.05-0.82) .02           |
|       | Lck-90       | 8/8                     | 1.26 (0.39-4.06) .70            | —                               |
|       | Lck-449      | 4/12                    | 0.69 (0.15-2.34) .57            | 0.60 (0.13-2.24) .46           |
|       | PAP-248      | 5/11                    | 3.84 (1.07-13.25) .04           | —                               |
|       | Lck-422      | 0/16                    | —                               | —                               |
|       | CypB-129     | 5/11                    | 1.61 (0.42-5.35) .46           | —                               |
|       | WHSC2-103    | 5/11                    | 0.53 (0.12-1.79) .32           | 0.44 (0.09-1.66) .23           |

Peptides used for <10% of patients were excluded from the univariate analysis. Correlated peptides are shown in italics and significant P-values are in shown in bold text. CI, confidence interval; HR, hazard ratio; UC, urothelial cancer; —, not applicable.
samples in this study. In a murine study, it was reported that inactivation of proximal T-cell receptor (TCR) signaling blocked by the inhibitory motif of p56Lck inhibited TIL lytic function.\textsuperscript{29} Tumor-induced lytic dysfunction of TIL may restrict T-cell–based cancer immunotherapy. Therefore, p56lck is one of the important targets for cancer immunotherapy. Similar to previous reports, PSA and PAP were not expressed in UC samples.\textsuperscript{30,31} However, ectopic expression of PSA has been reported in a variety of nonprostatic tumors, including UC of the bladder, breast cancer and melanoma.\textsuperscript{32-34} PSMA is a transmembrane receptor expressed on prostate cancer cells that is associated with a more aggressive phenotype. In the present study, PSMA was not expressed in some UC samples. However, a recent study indicated that PSMA is expressed in some subtypes of bladder cancer and is associated with tumor neovasculature based on IHC analysis.\textsuperscript{35} This report also suggested PSMA as a potential new vascular target for immunotherapy in UC patients.

\begin{table}
\centering
\caption{Peptide-specific IgG and CTL responses}\label{tab:5}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{HLA type} & \textbf{Peptide} & \textbf{No. of tested peptides} & \textbf{Immunological responses (%)} & & & \textbf{Advance UC} & \textbf{Immunological responses (\%)} \\
 & & & \textbf{IgG} & \textbf{CTL} & \textbf{No. of tested peptides} & \textbf{IgG} & \textbf{CTL} \\
\hline
A24 & SART2-93 & 49 & 3 (6) & 7 (14) & 29 & 6 (21) & 7 (24) \\
 & SART3-109 & 15 & 4 (27) & 2 (13) & 7 & 2 (29) & 0 \\
 & Lck-208 & 0 & — & — & 9 & 0 & 0 \\
 & PAP-213 & 12 & 4 (33) & 0 & 12 & 10 (83) & 6 (50) \\
 & PSA-248 & 29 & 19 (66) & 3 (10) & 11 & 5 (45) & 0 \\
 & EGF-R-800 & 19 & 1 (5) & 0 & 7 & 2 (29) & 3 (43) \\
 & MRP3-503 & 1 & 1 (100) & 0 & 0 & — & — \\
 & MRP3-1293 & 6 & 0 & 0 & 5 & 0 & 0 \\
 & SART2-161 & 10 & 3 (30) & 1 (10) & 3 & 0 & 1 (33) \\
 & Lck-486 & 23 & 8 (35) & 1 (4) & 21 & 7 (33) & 4 (19) \\
 & Lck-488 & 40 & 6 (15) & 6 (15) & 26 & 5 (19) & 4 (15) \\
 & PSMA-624 & 2 & 0 & 0 & 5 & 1 (20) & 2 (40) \\
 & EZH2-735 & 2 & 0 & 0 & 1 & 1 (100) & 1 (100) \\
 & PTHrP-102 & 7 & 3 (43) & 2 (29) & 7 & 1 (14) & 1 (14) \\
A2 & CypB-129 & 5 & 2 (40) & 0 & 9 & 2 (22) & 3 (33) \\
 & Lck-246 & 16 & 5 (31) & 2 (13) & 7 & 0 & 5 (71) \\
 & Lck-422 & 0 & — & — & 1 & 0 & 0 \\
 & ppMAPkkk-432 & 8 & 0 & 0 & 2 & 0 & 0 \\
 & WHSC2-103 & 12 & 4 (33) & 1 (8) & 4 & 0 & 1 (25) \\
 & HNRPL-501 & 8 & 5 (63) & 0 & 3 & 2 (67) & 2 (67) \\
 & UBE2V-43 & 6 & 5 (83) & 0 & 7 & 3 (43) & 2 (29) \\
 & UBE2V-85 & 0 & — & — & 0 & — & — \\
 & WHSC2-141 & 10 & 4 (40) & 1 (10) & 7 & 2 (29) & 2 (29) \\
 & HNRPL-140 & 5 & 2 (40) & 1 (20) & 1 & 0 & 0 \\
 & SART3-302 & 10 & 5 (50) & 1 (10) & 6 & 4 (67) & 1 (17) \\
 & SART3-309 & 9 & 3 (33) & 0 & 3 & 1 (33) & 0 \\
A3 & SART3-109 & 4 & 2 (50) & 1 (25) & 3 & 1 (33) & 0 \\
 & SART3-511 & 9 & 1 (11) & 0 & 8 & 1 (13) & 1 (13) \\
 & SART3-734 & 14 & 2 (14) & 1 (7) & 10 & 1 (10) & 2 (20) \\
 & Lck-90 & 13 & 4 (31) & 2 (15) & 6 & 2 (33) & 2 (33) \\
 & Lck-449 & 3 & 1 (33) & 0 & 4 & 3 (75) & 0 \\
 & PAP-248 & 3 & 1 (33) & 0 & 5 & 1 (20) & 0 \\
 & Lck-422 & 0 & — & — & 0 & — & — \\
 & CypB-129 & 3 & 0 & 0 & 4 & 0 & 0 \\
 & WHSC2-103 & 8 & 2 (25) & 0 & 4 & 1 (25) & 1 (25) \\
\hline
\end{tabular}
\end{table}

CRPC, castration-resistant prostate cancer; CTL, cytotoxic T lymphocyte; PPV, personalized peptide vaccine; UC, urothelial cancer; —, not applicable.
Selection of clinically effective peptides is essential for peptide-based cancer vaccines. In PPV treatment, up to 4 different peptides from 31 candidate peptides are given to individual patients based on HLA type and pre-existing immunity by measuring peptide-specific IgG levels, and the clinical benefits of PPV for advanced urological cancers have been reported. However, it is important to discriminate more clinically effective peptides among IgG-positive peptide candidates prior to PPV treatment. To select peptides correlated with clinical efficacy, univariate and multivariate analyses with Cox regression hazards models were carried out. In the present study, patients with the following peptides had a significantly longer survival than patients without them (HR < 1.0, 95% CI < 1.0 and \( P < .05 \)): SART3-109 (for HLA-A24), PTHrP-102 (for HLA-A24), HNPRL-140 (for HLA-A2), SART3-302 (HLA-A2) and Lck-90 (for the HLA-A3 family) in CRPC patients, and EGF-R-800 (for HLA-A24), Lck-486 (for HLA-A24), PSMA-624 (for HLA-A24), CypB-129 (for HLA-A2) and SART3-734 (for the HLA-A3 family) in advanced UC patients, respectively. These peptides induced peptide-specific IgG and CTL responses and were considered to be peptides correlated with clinical benefits. In addition, the median OS times for both CRPC and advanced UC patients treated by PPV including correlated peptides were significantly longer than for those treated without correlated peptides. Selection of multiple epitopes for PPV treatment may reduce the risk of tumor escape through the existence and/or induction of antigen-negative clones escaping peptide-specific immune responses. It is relatively rare for tumor cells to escape peptide-specific immune responses by simultaneously losing all antigens selected for vaccination.

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In conclusion, the use of correlated peptides selected by both survival data and pre-existing immunity for PPV treatment may enhance the clinical benefits for urological cancer patients. To confirm these results, a clinical trial of novel PPV treatment for urological cancer patients is needed in which the peptides used for novel PPV treatment are first selected from correlated peptides and then up to 4 peptides are selected based on pre-existing immunity according to HLA type.

CONFLICTS OF INTEREST

Noguchi M has served as an advisory board consultant for BrightPath Bio. Co. Ltd. Itoh K has served as a consultant and received research funding from Taiho Pharmaceutical Company. Yamada A is a part-time executive of BrightPath Bio. Co. Ltd and has stock in this company. Koga N, Moriya F, Suekane S, Yutani S, Shichijo S and Kakuma T declare no competing interests.

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FIGURE 2  Kaplan-Meier curves comparing overall survival with or without CP among CRPC (A) and advanced UC patients (B). CP, correlated peptide; CRPC, castration-resistant prostate cancer; HLA, human leukocyte antigen; MST, median survival time; UC, urothelial cancer
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