

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 into 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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RESEARCH ARTICLE

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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

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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

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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	ASLSDPWV	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	TFDYLRSLV	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	QIRPFSNR	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

^aA3sup, 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/ μ L	
Low	41
High	59
CRP, 3 μ g/mL	
Low	53
High	47
SAA, 8 μ g/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

Table 2 Patient characteristics (Continued)

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.

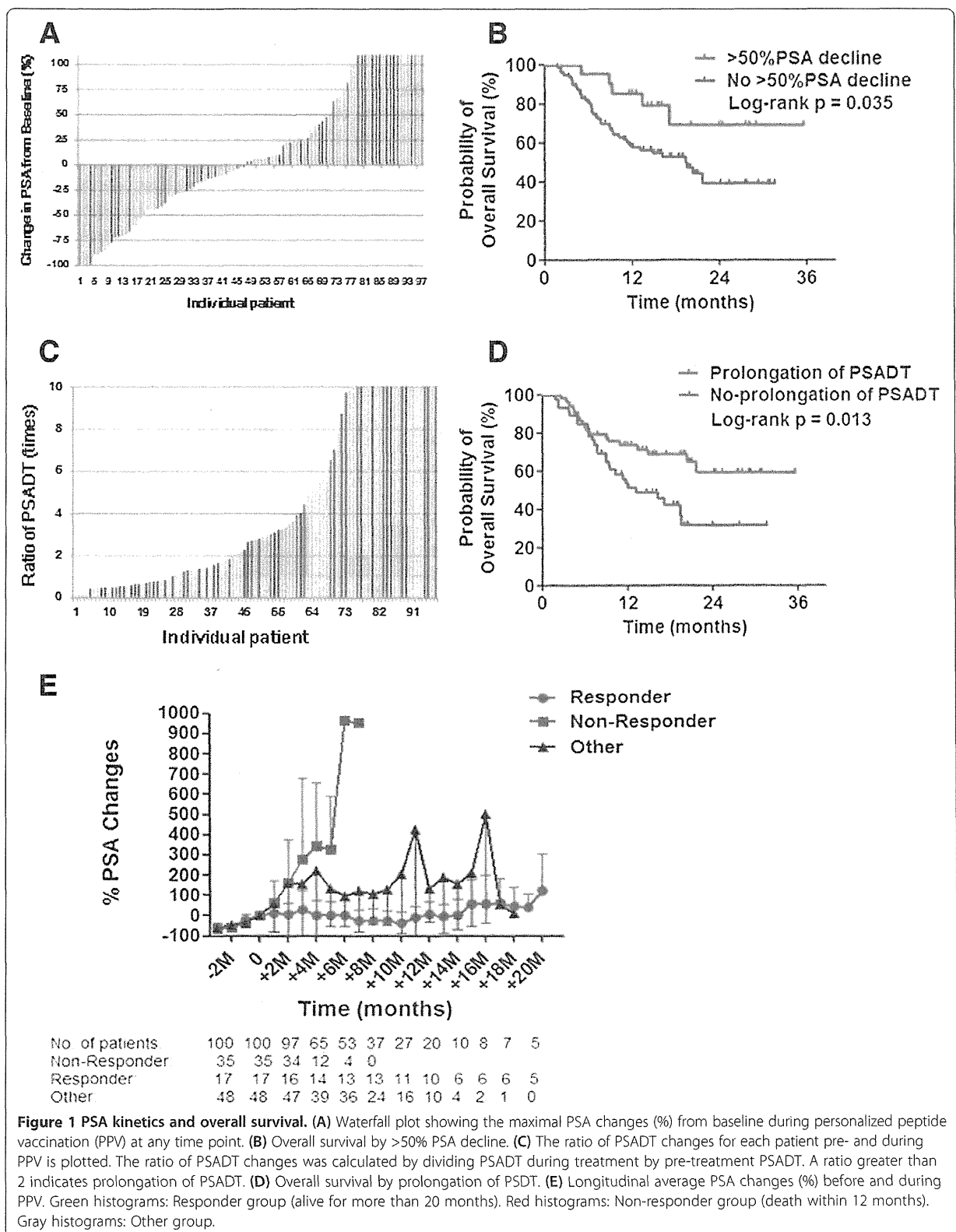
Although T-cell subsets using flowcytometry was not analyzed in this study, T-cell responses specific to the vaccinated peptides were evaluated by IFN- γ ELISPOT assay using peripheral blood mononuclear cells (PBMCs), as reported previously [18]. Peptide-specific T-cell responses were evaluated by the 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



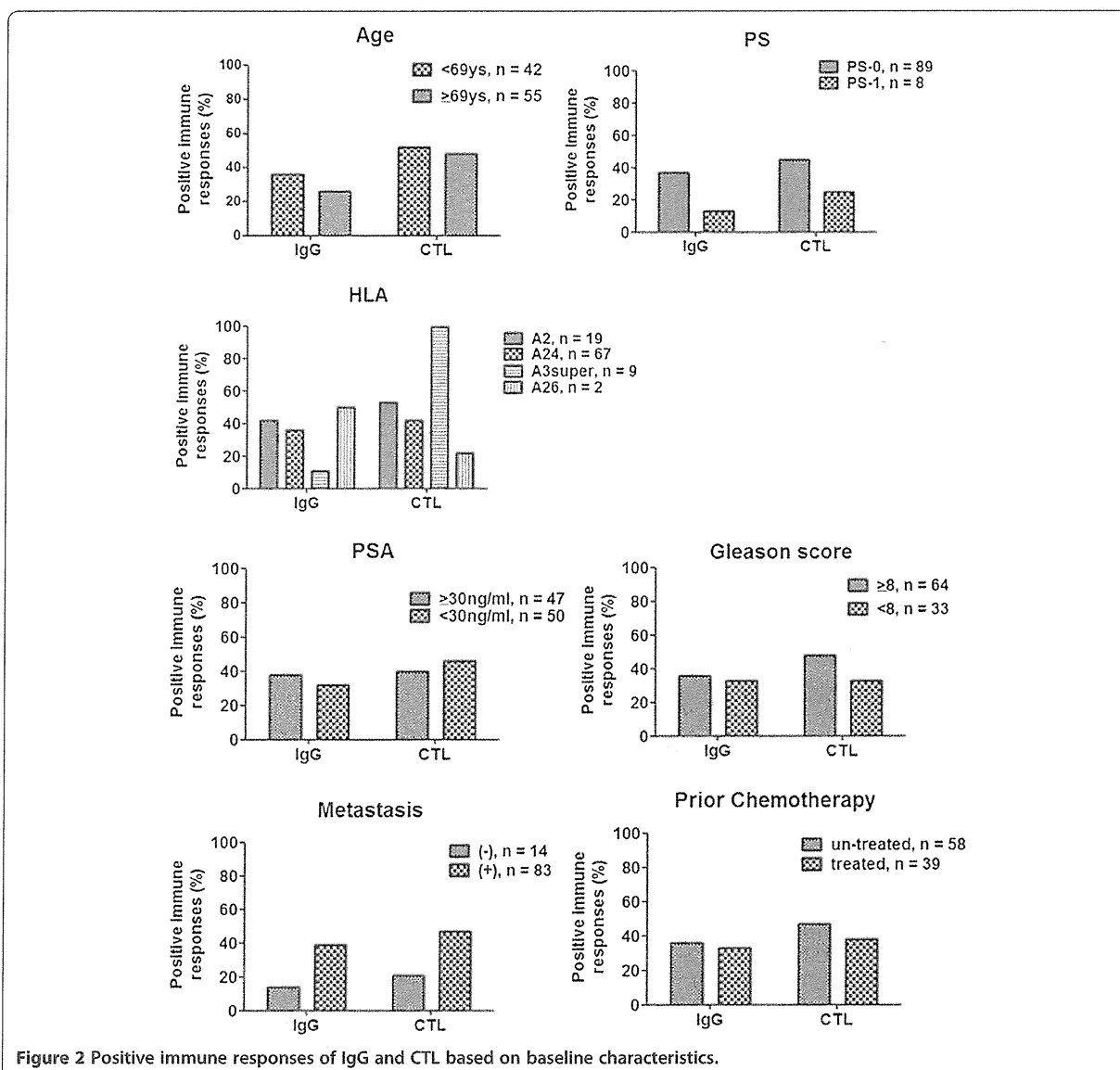
analyzed at the end of November, 2012 using commercially available computer software. The Student's t-test and the chi-square test were used to compare quantitative and categorical variables, respectively. Survival was calculated from the date of first treatment until the date of any cause of death. 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



median age of participants was 69 years (range, 51 to 92 years), and the ECOG performance status was 0 in 91 of 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

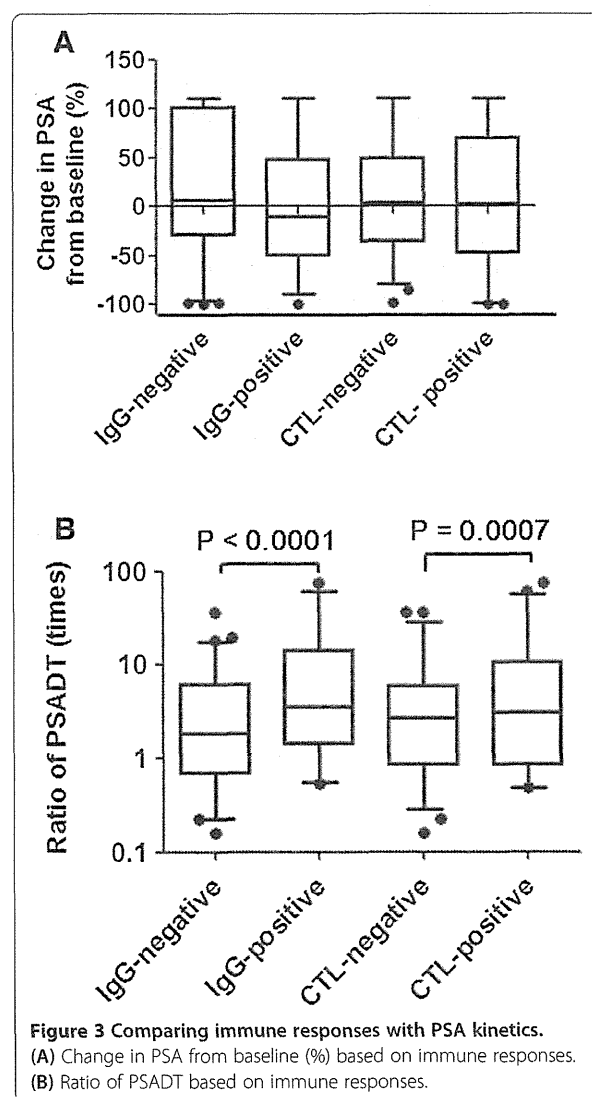
The overall toxicities are shown in Table 3. The most frequent adverse events were local redness and swelling at injection sites, bone pain, hypoalbuminemia, lymphocytopenia, appetite loss, fatigue, increased ALP, and anemia, which were grade 1 or 2 in most cases. There were no grade 4 toxicities and 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 $\geq 50\%$ PSA decline at any point during PPV was observed in 21 patients (22%), with a median time of 4 months to $\geq 50\%$ PSA decline and a median duration of $\geq 50\%$ PSA decline of 3 months. Delayed PSA response was observed. Patients with $\geq 50\%$ 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.



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 $\geq 50\%$ 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/ μ L) 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 μ g/mL) vs. high	0.709	0.875	0.433-1.767	-	-	-

Of the 100 men, 64 died.

^aLymphocyte, 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 responses, 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 manuscript. 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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Current status of immunotherapy for the treatment of biliary tract cancer

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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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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.

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-naïve 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 super-types (-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 administered (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.

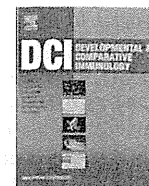
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Humoral immune responses to CTL epitope peptides from tumor-associated antigens are widely detectable in humans: A new biomarker for overall survival of patients with malignant diseases



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ABSTRACT

Both cellular and humoral immune responses are crucial to induce potent anti-tumor immunity, but most of currently conducted peptide-based cancer vaccines paid attention to cellular responses alone, and none of them are yet approved as a therapeutic modality against cancer patients. We investigated humoral immune responses to CTL epitope peptides derived from tumor-associated antigens in healthy donors and patients with various diseases to facilitate better understanding of their distribution patterns and potential roles. Bead-based multiplex assay, ELISA, and Western blotting were used to measure immunoglobulins reactive to each of 31 different CTL epitope peptides. Importantly, the sums of anti-peptide IgG levels specific to 31 CTL epitope peptides were well correlated with better overall survival (OS) in patients with malignant diseases. Our results suggested that humoral immune responses to CTL epitope peptides were widely detectable in humans. Measurement of immunoglobulins specific to CTL epitope peptides may provide a new biomarker for OS of patients with malignant diseases, although it still remains to be determined whether the correlations between humoral immune responses to epitope peptides and OS are observed only for the CTL epitopes used, or also for other panels of peptides. Quantity of circulating IgG reactive to these peptides was also discussed.

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1. Introduction

Peptide-based cancer vaccines have been extensively studied following the discovery of human tumor-associated antigens (TAA) and cytotoxic T lymphocyte (CTL) epitope peptides

(Rosenberg et al., 2004; Mellman et al., 2011). However, none of them are yet approved as a therapeutic modality. There might be at least two important hurdles to obtain clinical benefits from the peptide-based cancer therapies currently in practice. One of these hurdles, the negative signaling against CTL activation through check point molecules, such as CTLA-4 and PD-1, was recently overcome by developing blocking antibodies against these molecules (Hodi et al., 2010; Topalian et al., 2012; Brahmer et al., 2012). The second potential hurdle is that no or little humoral immune responses can be induced by the vaccination using most of currently available CTL epitope peptides, although it has been well recognized that both cellular and humoral immune responses are

Abbreviations: TAA, tumor-associated antigen; HD, healthy donor; Flu, influenza virus; HCV, hepatitis C virus; Ig, immunoglobulin; OS, overall survival.

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crucial to induce potent anti-tumor immunity in animal models (Hu et al., 2005; Bequet-Romero et al., 2012; Zeng et al., 2009). In fact, most of currently conducted peptide-based cancer vaccines have paid attention to cellular immune responses alone. To our knowledge, exception is “personalized peptide vaccination” that we have developed (Terasaki et al., 2011), in which CTL epitope candidates for therapeutic cancer vaccines were at first screened based on not only their ability to induce CTL but also reactivity to IgG responses in pre-vaccination samples.

Although humoral immune responses against whole proteins of TAA have been well investigated (Yuan et al., 2011; Toh et al., 2009; Zhang and Tan, 2010), those against CTL epitope peptides derived from TAA, which have been used for therapeutic cancer vaccines, have rarely been studied. We hypothesized that a CTL epitope peptide possessing a B cell epitope could provide more effective clinical benefits than a CTL epitope peptide without it. In fact, we reported potential clinical benefits in advanced glioblastoma multiforme or prostate cancer patients under personalized peptide vaccines using such peptides (Terasaki et al., 2011; Noguchi et al., 2010, 2011a,b; Yajima et al., 2005). In addition, IgG responses were identified as an excellent prognostic marker for predicting overall survival (OS) of the vaccinated patients, although CTL responses also showed a prognostic correlation (Noguchi et al., 2011b; Mine et al., 2004). However, it remains to be fully studied whether anti-peptide immunoglobulins (Igs) are detectable in healthy donors (HD) and patients with various diseases. The current study has addressed this issue to facilitate better understanding of humoral immune responses to CTL epitopes and better designing of cancer vaccine protocols. The results suggest that humoral immune responses are widely detectable in humans and have potential as a new biomarker for overall survival (OS) of patients with malignant diseases.

2. Materials and methods

2.1. Patients and sample collection

Plasma or sera were collected from HD ($n = 74$, 43 ± 20 years old) and from patients with rheumatoid arthritis ($n = 20$, 67 ± 7 years old), IgA nephropathy ($n = 20$, 34 ± 13 years old), influenza virus (Flu) infection ($n = 20$, 34 ± 17 years old), hepatitis C virus (HCV) infection ($n = 20$, 55 ± 8 years old), hematological malignancies ($n = 55$, 61 ± 14 years old; 24 leukemia, 27 lymphoma, and 4 myeloma), or non-HCV hepatocellular carcinoma (HCC, $n = 55$, 60 ± 11 years old; 26 non-B non-C hepatocellular carcinoma, 23 hepatitis B associated hepatocellular carcinoma and six alcoholic hepatic carcinoma) (Supplementary Table 1). HD were categorized into the following four age groups according to the Ministry of Health, Labor and Welfare in Japan; 15–24 ($n = 20$), 25–44 ($n = 19$), 45–64 ($n = 23$), and ≥ 65 ($n = 12$) years old (Supplementary Table 2). This study was approved by the Kurume University Ethical Committee. After informed consent was obtained from all subjects, blood samples (plasma or sera) were obtained and frozen at -80°C until use (Noguchi et al., 2011b; Mine et al., 2004).

2.2. Peptides

Thirty-one different peptides employed in the current study were prepared under conditions of Good Manufacturing Practice by Poly Peptide Laboratories (San Diego, CA) or American Peptide Company (Vista, CA), and dissolved in DMSO (Wako, Osaka, Japan). Detailed information on these peptides, including the original protein, peptide position, amino acid sequence, HLA class I A restriction, and references, are given in Supplementary Table 3. Twenty-four of 31 peptides were derived from TAA that were

identified by the cDNA expression cloning method, followed by determination of CTL epitopes. The remaining seven peptides including PAP-213, PSA-248, PSMA-624, and PAP-248 were identified by the reverse-immunology method (Kobayashi et al., 2003; Matsueda et al., 2005; Inoue et al., 2001). CTL epitope peptides were determined to be cancer vaccine candidates, based on both their ability to induce CTL activity from peripheral blood mononuclear cells *in vitro* as well as the IgG levels against them in plasma of un-vaccinated cancer patients, and these peptides have been used in clinical trials of personalized peptide vaccine for advanced cancer patients (Terasaki et al., 2011; Noguchi et al., 2010, 2011a,b; Yajima et al., 2005; Mine et al., 2004; Terazaki et al., 2012).

2.3. Measurement of Igs reactive to each of 31 different peptides

The levels of Igs reactive to each of 31 different peptides were measured by multiplex bead suspension array using the Luminex system (Luminex Corp., Austin, TX) as reported previously (Komatsu et al., 2004). In brief, plasma or serum was incubated with 100 μL of peptide-coupled color-coded beads for 1.5 h at 30°C . To detect IgG or IgM, after washing, the beads were incubated with 100 μL of biotinylated goat anti-human IgG (gamma chain-specific; Vector Laboratories, Burlingame, CA) or biotinylated goat anti-human IgM (mu chain-specific; Vector Laboratories) Abs for 1 h at 30°C . To detect IgG1, IgG2, IgG3, or IgG4, the beads were incubated with 100 μL of sheep anti-human IgG1, IgG2, IgG3, or IgG4 Abs (Binding Site, Birmingham, UK) for 1 h at 30°C , followed by washing and incubation with 100 μL biotin-rabbit anti-sheep IgG Ab for 1 h at 30°C . After washing, the beads were incubated with 100 μL of streptavidin-PE (Life Technologies, Carlsbad, CA) for 30 min at 30°C , followed by washing and detection of fluorescence intensity unit (FIU) on the beads using the Luminex system (Komatsu et al., 2008). The cut-off values of anti-peptide IgG were set to 10 FIU in 100-time diluted samples, as reported previously (Komatsu et al., 2008). In brief, the calibration curves of FIU were obtained with serially diluted samples. The plasma samples from cancer patients were two times diluted from 160 to 1,310,720. The minimum detectable level of anti-peptide IgGs was 2 FIU when the samples were diluted at 40,960 times as shown by an arrow in Supplementary Fig. 1. However, the levels of anti-peptide IgGs at the minimum detectable range were not reliable since the standard deviations were high. Therefore, we set 10 FIU of the 100-time diluted sample, which was considered to be a reliable value, as a cut-off level as reported previously (Komatsu et al., 2008). There were no significant differences between plasma and serum with regard to the levels of anti-peptide Igs (data not shown).

The specificities of IgG against these peptides were confirmed by competition assay. Plasma was incubated with 100 μL of peptide-coupled color-coded beads and 5 μL of each of the corresponding peptides for 1.5 h at 30°C . The binding of anti-peptide IgG was detected by same method as described above.

Plasma from frequently vaccinated (12–18 vaccinations) cancer patients who were enrolled in clinical trials of personalized peptide vaccine (data not shown) were used for estimation of anti-peptide IgG levels by other methods, Western blotting and ELISA. To isolate anti-peptide IgGs for Western blotting, plasma was incubated with 100 μL of peptide-coupled color-coded beads for 1.5 h at 30°C . After washing, the beads were incubated with 6 μL of sample buffer (NuPAGE LDS Sample buffer; Life Technologies, Carlsbad, CA) at 70°C for 15 min prior to loading onto the SDS-PAGE gel. The separated proteins were transferred to nitrocellulose membrane (Life Technologies, Carlsbad, CA), and IgG gamma chain was detected by using Goat F(ab')₂ Fragment anti-human IgG(H+L)-peroxidase (IM0837; Beckman Coulter, Fullerton, CA) Ab and an ECL system (GE Healthcare, Uppsala, Sweden). As a

standard, purified human IgG (R&D Systems, Minneapolis, MN) was used. ImageQuant ver 5.2 (GE Healthcare, Pittsburgh, PA) was used to measure chemi-luminescent signals for quantification.

A human IgG ELISA kit (Bethyl Laboratories Inc., Montgomery, TX) was also used for quantitative analysis of anti-peptide IgGs in plasma from frequently vaccinated patients, which were also used for Western blotting. One hundred-time diluted plasma was incubated with 100 μ L of peptide-coupled color-coded beads for 1.5 h at 30 °C. After centrifugation, the supernatant was added to new beads, and incubated again to detect the remaining anti-peptide IgGs in plasma. The beads were washed and incubated with 90 μ L of 0.1 M Glycine buffer (pH 2.7) for 5 min at 30 °C, and then 10 μ L of 1 M Tri-HCl (pH 9.0) buffer was added to neutralize eluted fraction. After centrifugation, the supernatant was collected for quantitative analysis by ELISA. The optical density was determined using a microplate reader (Infinite[®] 200; TECAN, Männedorf, Switzerland).

2.4. Statistical analysis

The *t*-test and the Chi-square test were used to determine whether there is a significant difference in age or gender. Wilcoxon signed rank test was used to compare Ig levels specific to peptides. The OS in cancer patients was calculated from the date of drawing blood until the date of death or the last date when the patient was known to be alive. Curves for OS were estimated by the Kaplan–Meier method, and the log-rank test was conducted for the comparison of survival curves. A two-sided *P* value of less than 0.05 was considered to be statistically significant. All statistical analyses were conducted by using the JMP version 9.1 software (SAS Institute Inc., Cary, NC).

3. Results

3.1. Detection of Igs specific to CTL epitope peptides in HD

We first addressed whether Igs reactive to each of 31 different CTL epitope peptides derived from TAA were detectable in plasma or sera from HD (*n* = 74) by the Luminex system. There are no significant differences in Ig levels measured between in sera and in plasma (data not shown). IgM reactive to all but two (Lck-422 and MRP3-503) of the 31 peptides were detected as positive in >50% of HD, since their median values exceeded the cut-off values (10 FIU) (Table 1). Similarly, IgGs reactive to 23 peptides, but not to the remaining eight peptides (Lck-422, ppMAPkkk-432, and the others), were detected as positive in >50% of HD. IgG1, IgG2, IgG3, and IgG4 levels were also detected as positive in >50% of HD in 22, 15, 12, or 0 of 31 peptides, respectively (Table 1).

The specificities of IgG reactive to nine peptides were previously reported (Kobayashi et al., 2003; Matsueda et al., 2005; Harada et al., 2003; Shomura et al., 2004; Ogata et al., 2004; Yao et al., 2004; Minami et al., 2007). The specificities of IgG against the remaining 22 peptides were confirmed in this study by competition assays, in which the binding of anti-peptide IgG was inhibited in the presence of each of the corresponding peptides, and representative results for the 12 peptides are shown in Supplementary Fig. 2.

We next examined the effects of gender and age on the anti-peptide Ig levels. There were no significant differences between males (*n* = 41) and females (*n* = 33) with regard to the levels of IgM, IgG, IgG1, IgG2, IgG3, or IgG4 against any of the 31 peptides, or the sums of the Igs against each of the 31 peptides (data not shown). When the subjects were divided into the following four age groups: 15–24 years old (*n* = 20), 25–44 (*n* = 19), 45–64 (*n* = 23), and \geq 65 (*n* = 12) (Supplementary Table 2), there was an

Table 1

Assessment of immunoglobulins reactive to each of the 31 different CTL epitopes in plasma or sera from healthy donors.

Peptide name	HD (median FIU) ^a					
	IgM	IgG	IgG1	IgG2	IgG3	IgG4
CypB-129	350	20	19	<10	<10	<10
Lck-246	925	42	25	18	<10	<10
Lck-422	<10	<10	<10	<10	<10	<10
ppMAPkkk-432	103	<10	<10	<10	<10	<10
WHSC2-103	4940	114	69	61	16	<10
HNRPL-501	71	<10	<10	<10	<10	<10
UBE2V-43	1696	51	50	10	25	<10
UBE2V-85	73	<10	<10	<10	<10	<10
WHSC2-141	764	49	36	25	<10	<10
HNRPL-140	721	41	41	29	<10	<10
SART3-302	223	38	50	<10	<10	<10
SART3-309	2655	32	34	<10	11	<10
SART2-93	11,500	148	111	66	28	<10
SART3-109	511	<10	<10	<10	<10	<10
Lck-208	654	11	11	<10	<10	<10
PAP-213	2134	48	34	<10	17	<10
PSA-248	2743	50	41	10	12	<10
EGFR-800	2659	58	39	25	<10	<10
MRP3-503	<10	<10	<10	<10	<10	<10
MRP3-1293	1376	48	37	15	12	<10
SART2-161	84	<10	<10	<10	<10	<10
Lck-486	1277	65	49	<10	17	<10
Lck-488	6302	135	111	35	41	<10
PSMA-624	426	23	19	<10	<10	<10
EZH2-735	261	<10	<10	<10	<10	<10
PTHrP-102	1467	13	22	24	<10	<10
SART3-511	5057	70	60	19	17	<10
SART3-734	2302	188	164	74	23	<10
Lck-90	7410	116	90	34	25	<10
Lck-449	590	44	33	<10	<10	<10
PAP-248	420	29	<10	13	<10	<10

^a IgM, IgG, and IgG subclasses (IgG1, IgG2, IgG3, and IgG4) specific to each of the 31 CTL epitope peptides were measured by the Luminex system in 100-time diluted samples (plasma or sera) from healthy donors (HD). The median values of FIU are shown.

age-dependent decrease of IgM levels against each of the 31 peptides (data not shown) or of the total sum of the IgM against each of the 31 peptides (Fig. 1). A similar trend was also observed for the levels of anti-peptide IgG as well as for those of anti-peptide IgG subclasses, including IgG1 and IgG2, whereas IgG and IgG1 levels were somewhat increased in the oldest age bracket (\geq 65 years old) (Fig. 1).

3.2. Detection of Igs specific to CTL epitope peptides in patients with various diseases

We next examined the levels of anti-peptide Igs in patients with various types of immune-related disorders, including autoimmune diseases, immune-complex-related diseases, and acute and chronic infectious diseases (Supplementary Table 1). The total sums of Igs against each of 31 peptides from patients' samples were compared with those from the age- and gender-matched HD samples. We measured anti-peptide Igs in patients with rheumatoid arthritis and IgA nephropathy as an example of autoimmune diseases and immune-complex-related diseases, respectively. There were no significant differences between patients with rheumatoid arthritis and HD with regard to either Ig levels to each peptide or the total sums of them (Fig. 2A). In contrast, the total sums of anti-peptide IgM (*P* < 0.0001) and IgG2 (*P* = 0.0142) in patients with IgA nephropathy were significantly lower than those in HD, respectively (Fig. 2B). In addition, we measured anti-peptide Igs in patients with Flu infection and HCV infection as an example of acute and chronic viral infections, respectively. The total sums of anti-peptide IgM in patients with Flu infection were significantly