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Feasibility study of personalized peptide vaccination for advanced non-small cell lung cancer patients who failed two or more treatment regimens

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Abstract. The prognosis of non-small cell lung cancer (NSCLC) patients who failed two or more treatment regimens remains very poor. We conducted a phase II study to explore the feasibility of personalized peptide vaccination (PPV), in which peptides are selected and administered based on the pre-existing host immunity before vaccination, as a third or more line treatment in advanced NSCLC patients who failed two or more regimens. Among 57 patients enrolled, 23 or 16 patients received PPV with chemotherapy or targeted therapy, respectively, whereas 18 patients received PPV alone. A maximum of four HLA-matched peptides showing higher peptide-specific IgG responses in pre-vaccination plasma were selected from 31 pooled peptide candidates applicable for patients with HLA-A2, -A24, -A3 supertypes, and/or -A26, followed by subcutaneous administration. No severe adverse events related to PPV were observed. Median survival time was 692, 468, or 226 days in the group of PPV/chemotherapy, PPV/targeted therapy, or PPV alone, respectively. CTL responses to the vaccinated peptides became detectable after vaccination in 58, 50, or 42% of patients in each of these three groups, respectively. In contrast, peptide-specific IgG responses after vaccination augmented in 55, 75, or 62% of patients in each of these groups, respectively. These results suggest the feasibility of PPV for heavily treated advanced NSCLC patients from the view of both immunological responses and safety. Therefore, further evaluation of PPV by prospective randomized trial is warranted for a third or fourth line treatment of advanced NSCLC.

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Abbreviations: NSCLC, non-small cell lung cancer; PPV, personalized peptide vaccination; OS, overall survival; IHC, immunohistochemistry; SCC, squamous cell carcinoma; ECOG, Eastern Cooperative Oncology Group; HLA, human leukocyte antigen; NCI-CTC, National Cancer Institute Common Terminology Criteria for Adverse Events; CTL, cytotoxic T lymphocyte; IFN, interferon; PBMCs, peripheral blood mononuclear cells; SAEs, severe adverse events; MST, median survival time

Key words: non-small cell lung cancer, peptide vaccine, personalized medicine, clinical study, third line therapy

Introduction

Lung cancer is the leading cause of cancer-related deaths globally, and non-small cell lung cancer (NSCLC) is the most common type, observed in approximately 85% of patients, making it a major global public health concern (1,2). Despite dramatic advances in the treatment of NSCLC over the last two decades, most of the patients experience disease progression and succumb to the disease. Since the prognosis of refractory NSCLC patients who failed two or more treatment regimens remains very poor (3-7), development of newer therapeutic approaches are needed. One of the new approaches might be the blockade of T cell inhibition mediated by checkpoint molecules, such as CTLA-4, PD-1, and PD-L1 in NSCLC patients (8-11). The other might be a personalized approach, and we have developed a novel regime of personalized peptide vaccination (PPV), in which peptides are selected and administered

based on the pre-existing host immunity before vaccination (12-17). PPV could have the potential to prolong overall survival (OS), but not progression-free survival, in advanced cancer patients who failed standard chemotherapy (12-16). We also reported that high level of plasma C-reactive protein was a significant predictor of unfavorable OS in refractory NSCLC patients (17). In the present study, we investigated the feasibility of PPV as a third or fourth line therapy for NSCLC patients.

Patients and methods

Immunohistochemistry (IHC). Expression of 15 vaccine antigens, from which the peptides were derived, was examined by IHC in primary cancer tissues of 20 non-vaccinated NSCLC patients [10 adenocarcinoma and 10 squamous cell carcinoma (SCC)] that were obtained at the time of radical operation. Paraffin-embedded tissue samples were cut into 4-μm sections, and examined on a coated slide glass. Detailed methods including antibodies used for IHC were previously described (15).

Patients. Patients diagnosed as advanced NSCLC patients who failed two or more treatment regimens were eligible to this study. All patients were required to have been diagnosed as stage IIIB, IV or recurrent at the time of entry. They had to show positive IgG responses to at least two of the 31 different vaccine candidate peptides, as reported previously (13-17). Other inclusion criteria were as follows: age between 20 and 80 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 at the time of first visit; positive status for human leukocyte antigen (HLA)-A2, -A24, -A3 supertypes (A3, A11, A31, or A33), or -A26 types; life expectancy of ≥12 weeks; and adequate hematologic, hepatic, and renal function. Exclusion criteria included pulmonary, cardiac, or other systemic diseases; an acute infection; a history of severe allergic reactions; pregnancy or nursing; and other inappropriate conditions for enrollment as judged by clinicians. The protocol was approved by the Kurume University Ethics Committee and registered in the UMIN Clinical Trials Registry (UMIN nos. 1482, 1839 and 2984). All patients were given a full explanation of the protocol and provided their informed consent before enrollment. Two patients whose performance status were evaluated as 2 at the time of 1st vaccination (two weeks after the first visit), were excluded from this study, while three patients who did not agree to combined chemotherapy or targeted therapy regardless of their tolerability were also excluded from this study.

Clinical protocol. This was a phase II study to evaluate the safety, immunological responses, and clinical benefits from a view of OS in heavily treated advanced NSCLC patients under PPV. Thirty-one peptides were employed for vaccination [12 peptides for HLA-A2, 16 peptides for HLA-A24, 9 peptides for HLA-A3 supertypes (-A3, -A11, -A31, and -A33), and 4 peptides for HLA-A26] as reported previously (13-17). These peptides were prepared under the condition of Good Manufacturing Practice by the PolyPeptide Laboratories (San Diego, CA, USA) and American Peptide Co. (Vista, CA, USA). Peptides for vaccination to individual patients were selected in consideration of the pre-existing host immunity before vaccination, as assessed by the titers of IgG specific to each of the 31 different vaccine candidates (18,19). A maximum of 4 peptides (3 mg/each peptide), which were selected based on the results of HLA typing and peptide-specific IgG titers, were subcutaneously administered with incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France) once a week for 6 consecutive weeks (protocol nos. 1482 and 1839), or once a week for 4 consecutive weeks followed by biweekly administration 4 times (protocol no. 2984), as the 1st cycle. After the 1st cycle of vaccinations, up to 4 antigen peptides that were re-selected according to the titers of peptide-specific IgG were administered biweekly for 6 or 8 times, respectively. After the 2nd cycle of vaccinations, up to 4 antigen peptides that were re-selected again were administered every 4 weeks until 24th vaccination. During the PPV, patients received combination chemotherapy or targeted therapy (gefetinib, erlotinib, or crizotinib), unless they were unable to tolerate either therapy. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI-CTC Ver. 3.0). Complete blood counts and serum biochemistry tests were performed before and after each cycle of vaccinations.

Measurement of IgG and cytotoxic T lymphocyte (CTL) responses. Humoral immune responses specific to each of the 31 peptide candidates were determined by peptide-specific IgG levels using the Luminex system (Luminex, Austin, TX, USA), as previously reported (13-20). If the titers of peptide-specific IgG to at least one of the vaccinated peptides at the end of 1st cycle were >2-fold higher than those in the pre-vaccination plasma, the changes were considered to be significant as previously reported (13-18). CTL responses specific to the vaccinated peptides were evaluated by interferon (INF)-γ ELISPOT using peripheral blood mononuclear cells (PBMCs) before and at the end of 1st cycle as previously reported (13-18). As a control, CTL responses specific to CEF peptides (Mabtech, Cincinnati, OH, USA), a mixture of virus-derived CTL epitopes, were also examined.

Statistical analyses. All data were analyzed according to a pre-established plan. Comparison of each group was carried out by ANOVA test. OS was calculated from the first day of peptide vaccination until the date of death or the last date when the patient was known to be alive. The survival analysis was performed with the Kaplan-Meier method, and a comparison of the survival curves was performed with the log-rank test. If p-value was <0.05, it was considered as statistically significant. All statistical analyses were conducted using the JMP version 10 (SAS Institute Inc., Cary, NC, USA).

Results

Immunohistochemical analysis (IHC). The expression of 15 vaccine antigens for PPV was examined in 20 non-vaccinated NSCLC tissues (10 adenocarcinoma and 10 SCC). Representative results are shown in Fig. 1 (adenocarcinoma) and Fig. 2 (SCC). Twelve of 15 vaccine antigens were expressed at different frequencies in NSCLC tissues, as follows; Cyp-B:9/10, EGF-R:9/10, EZH2:9/10, HNRPL:10/10,

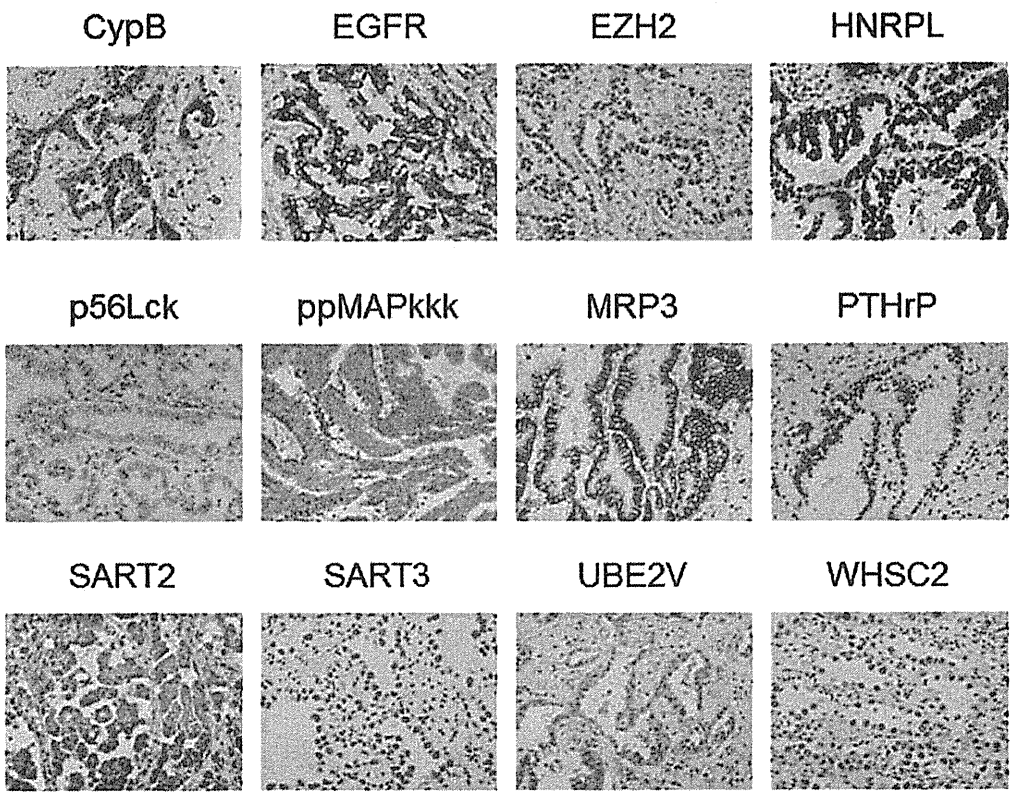

Figure 1. Expressions of vaccine antigen in lung adenocarcinoma tissues. Expression of 12 vaccine antigens, from which the peptides were derived, was examined by IHC in primary cancer tissues of 10 non-vaccinated lung adenocarcinoma patients. Representative results are shown (x400). The data of three prostate-related antigens, PAP, PSA and PSMA, are not shown.

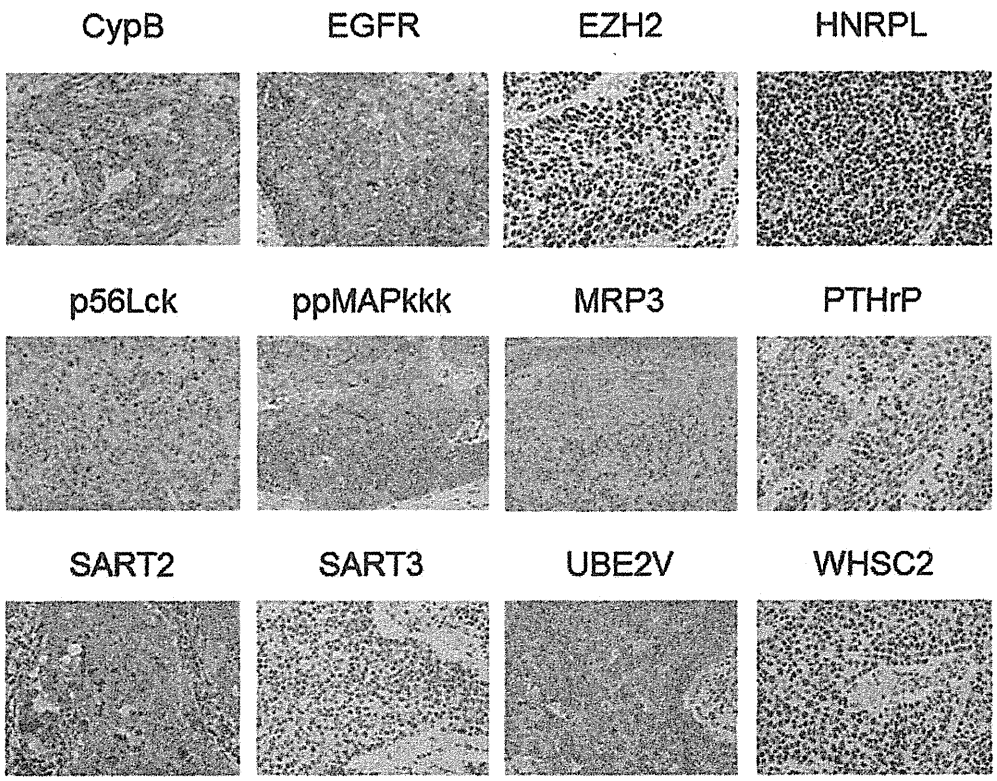

Figure 2. Expressions of vaccine antigen in lung SCC tissues. Expression of 12 vaccine antigens, from which the peptides were derived, was examined by IHC in primary cancer tissues of 10 non-vaccinated lung SCC patients. Representative results are shown (x400). The data of three prostate-related antigens, PAP, PSA and PSMA, are not shown.

Table I. The patient characteristics.

	Overall (n=57)	PPV/chemotherapy (n=23)	PPV/targeted therapy (n=16)	PPV alone (n=18)	p-value[a]
Age					0.010
Median (range)	64 (37-77)	64 (37-77)	57.5 (42-75)	67 (54-76)	
Sex					0.107
Male/female	27/30	13/10	4/12	10/8	
Performance status					0.079
0/1	25/32	12/11	9/7	4/14	
Histopathology					0.344
Adenocarcima	47	21	12	14	
Others	10	2	4	4	
Stage					0.838
IIIB/IV/recurrence	7/27/23	3/11/9	1/7/8	3/9/6	
HLA type					
HLA-A24	34	12	12	10	0.329
HLA-A2	22	8	7	7	0.852
Lymphocyte count					0.647
Median (range)	1,480 (1,014-3,399)	1,370 (1,014-2,653)	1,475 (1,058-3,192)	1,524 (1,032-3,399)	
No. of previous treatment regimen					0.076
Median (range)	3 (2-12)	4 (2-9)	3 (2-8)	4 (2-12)	
Time of peptide vaccination					0.093
Median (range)	11 (2-24)	13 (2-24)	13 (6-24)	8 (2-24)	

[a]ANOVA test was performed to examine p-values for continuous values or categorical values.

LCK:0/10, ppMAPkkk:10/10, MRP3:7/10, PTHrP:8/10, SART2:9/10, SART3:10/10, UBE2V:10/10, WHSC2:10/10 in adenocarcinoma tissues, and Cyp-B:9/10, EGF-R:10/10, EZH2:10/10, HNRPL:10/10, LCK:6/10, ppMAPkkk:8/10, MRP3:1/10, PTHrP:6/10, SART2:10/10, SART3:10/10, UBE2V:10/10, or WHSC2:9/10 in SCC tissues. Lck antigen, a unique vaccine antigen expressed in normal T cells and a part of metastatic tumor cells (21,22), was expressed in a small fraction of tumor cells in 0 of 10 adenocarcinoma, and 6 of 10 SCC tissues, respectively. None of the three prostate-related antigens (PAP, PSA and PSMA) were detectable in any of these tissues tested (data not shown).

The patient characteristics. Between December 2008 and May 2013, 57 patients with advanced NSCLC were enrolled to this study. Among them, 23 or 16 patients received PPV combined with chemotherapy or targeted therapy, respectively, whereas 18 patients did not tolerate either therapy and received PPV alone. The patient characteristics are shown in Table I. The PPV/targeted therapy group showed younger median age (p=0.010). Median number of previous treatment regimens before PPV in the groups of PPV/chemotherapy, PPV/targeted therapy, or PPV alone were 4, 3 or 4, respectively (p=0.076).

Adverse events. Median times of peptide vaccination were 11, ranging from 2 to 24 times (Table I). Table II shows severe adverse events (SAEs) during the PPV. Nine of 57 patients showed grade 3 SAEs (3 patients each in PPV/chemotherapy, PPV/target therapy and PPV alone group, respectively), and grade 4 SAEs occurred in 4 patients under PPV/chemotherapy. As the vaccination-related adverse events, almost all patients showed grade 1 or 2 dermatological reactions to PPV at the injection sites, but no patients showed SAEs (grade 3 or more) in agreement with previous reports (13-20).

Immune responses. Both peptide-specific CTL and IgG responses were analyzed in blood samples before and after the 1st cycle of vaccination. CTL responses to the vaccinated peptides were detectable in only 4/52 (7.7%) patients before vaccination (1, 2 and 1 patients under PPV/chemotherapy, PPV/targeted therapy, or PPV alone, respectively). However, it became detectable after the vaccination in 11/19 (58%), 7/14 (50%), or 5/12 (42%) patients in these groups, respectively (Tables III-V). We also tested CTL responses to CEF peptides, a mixture of virus-derived CTL epitopes, as a control. CTL responses to CEF peptides were observed in 14/42 (33.3%) patients before vaccination, and they were detectable after

Table II. Severe adverse events (grade 3 or 4) during the PPV.

	Overall (Grade 3/4)	PPV/chemotherapy (Grade 3/4)	PPV/targeted therapy (Grade 3/4)	PPV alone (Grade 3/4)
Constitutional symptom				
Fever	1/0	0/0	0/0	1/0
Tumor pain	1/0	0/0	1/0	0/0
Respiratory				
Dyspnea	1/0	0/0	1/0	0/0
Hypoxia	1/0	0/0	1/0	0/0
Neurological				
CNS cerebrovascular ischemia	0/1	0/1	0/0	0/0
Blood/bone marrow				
Anemia	1/1	0/1	0/0	1/0
Neutropenia	0/1	0/1	0/0	0/0
Lymphocytopenia	2/0	1/0	1/0	0/0
Thrombocytopenia	1/0	1/0	0/0	0/0
Metabolic and laboratory				
AST increased	1/0	1/0	0/0	0/0
ALT increased	1/0	1/0	0/0	0/0
γ-GTP increased	1/1	0/1	0/0	1/0
ALP increased	1/0	1/0	0/0	0/0

PPV, personalized peptide vaccination; CNS, central nervous system; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; ALP, alkaline phosphatase.

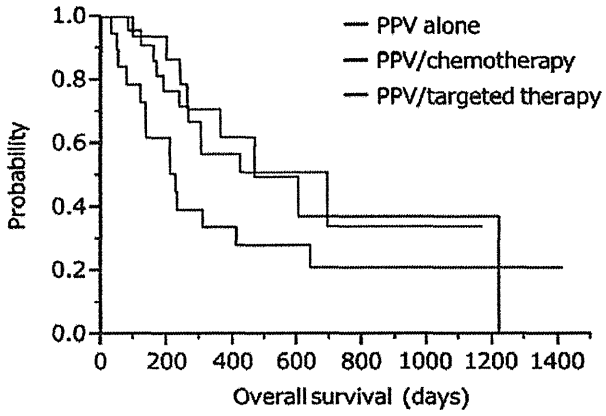

Figure 3. Survival analysis in the three subgroups. Median survival time (MST) was 692, 468, or 226 days in the group of PPV/chemotherapy, PPV/targeted therapy, or PPV alone, respectively. There was no significant difference among the three groups by the log-rank test (p=0.2475).

vaccination in 6/18 (33%), 3/9 (33%), or 6/9 (67%) patients in these groups, respectively (data not shown).

Peptide-specific IgG reactive to each of the 31 different peptides, including both vaccinated and non-vaccinated peptides, were measured by bead-based multiplex assay. IgG responses before vaccination were well observed in all the patients. IgG responses specific to at least one of the vaccinated peptides were increased after vaccination in 31/49 (63%) patients tested, with 11/20 (55%), 12/16 (75%), or 8/13 (62%) patients under PPV/chemotherapy, PPV/targeted therapy, or PPV alone group, respectively (Tables III-V). A greater number of peptides showed IgG responses to HLA-matched non-vaccinated peptides, but not to HLA-non-matched peptides, after vaccination in the PPV/chemotherapy group as compared to those in the PPV alone group (p=0.004) (data not shown).

Overall survival. Median survival time (MST) from the first vaccination of PPV was 692, 468, or 226 days in the group of PPV/chemotherapy, PPV/targeted therapy, or PPV alone, respectively (Fig. 3).

Discussion

It is important to better understand tumor immunity in refractory NSCLC patients who entered this study, since the repeated treatment regimens often suppress antitumor immunity. In addition, T cell checkpoint molecules, such as CTLA-4, PD-1, and PD-L1, were suggested to inhibit CTL responses against tumor cells in advanced cancer patients (8-11). As expected, CTL responses to the vaccinated peptides, but not to virus-derived peptides, before vaccination were rarely observed (1 of 22, 2 of 14, and 1 of 16 patients under PPV/chemotherapy, PPV/targeted therapy, or PPV alone, respectively), indicating antitumor immunity

Table III. Immune responses to peptides in the PPV/chemotherapy group.

Patient no.	No. of vaccinated peptide[a]	No. of peptides with enhanced IgG responses[b]	No. of peptides with enhanced CTL responses[c]
1	3	0	1
2	4	2	0
3	4	0	NA
4	4	0	1
5	4	NA	NA
6	4	0	1
7	4	1	0
8	4	3	1
9	4	1	2
10	4	NA	NA
11	4	2	0
12	4	0	1
13	4	2	1
14	4	0	0
15	4	1	0
16	4	0	0
17	4	0	1
18	4	2	0
19	4	NA	NA
20	4	4	2
21	4	2	0
22	4	3	1
23	4	0	1

PPV, personalized peptide vaccine; NA, not assessed. [a]Peptide numbers used for 1st cycle of vaccination. [b]If the titers of peptide-specific IgG at the end of 1st cycle were >2-fold higher than those in the pre-vaccination plasma, the changes were considered to be enhanced. [c]CTL responses were determined by the number of spots per 10^5 peripheral blood mononuclear cells (PBMCs) reactive with the vaccinated peptides in IFN-γ ELISPOT assay before and after 1st cycle of vaccination.

Table IV. Immune responses to peptides in the PPV/targeted therapy group.

Patient no.	No. of vaccinated peptide[a]	No. of peptides with enhanced IgG responses[b]	No. of peptides with enhanced CTL responses[c]
1	4	1	0
2	4	1	0
3	4	4	2
4	4	3	1
5	4	1	0
6	4	1	2
7	3	2	0
8	3	1	1
9	4	0	2
10	3	0	0
11	4	1	1
12	4	0	0
13	4	3	NA
14	4	1	1
15	4	0	0
16	4	2	NA

PPV, personalized peptide vaccine; NA, not assessed. [a]Peptide numbers used for 1st cycle of vaccination. [b]If the titers of peptide-specific IgG at the end of 1st cycle were >2-fold higher than those in the pre-vaccination plasma, the changes were considered to be enhanced. [c]CTL responses were determined by the number of spots per 10^5 peripheral blood mononuclear cells (PBMCs) reactive with the vaccinated peptides in IFN-γ ELISPOT assay before and after 1st cycle of vaccination.

of these patients was severely depressed. However, CTL responses to the vaccinated peptides became detectable at the end of the 1st cycle (6 or 8 times of vaccination) in 58, 50, or 42% of patients tested in these three groups, respectively. In addition, PPV did not affect CTL responses to virus-derived peptides. No PPV-related severe adverse events were observed in any of patients in this study, in agreement with the previous reports (13-20). These results suggest the feasibility of PPV for heavily treated advanced NSCLC patients who failed at least two regimens from the view point of both immunological responses and safety.

MST of patients under PPV/chemotherapy from the first vaccination of PPV was 692 days. Since the MST of the third or fourth line chemotherapy for refractory NSCLC patients was reported to be ~12 months or <12 months, respectively (23-25), the current data might be promising. MST of patients under PPV/targeted therapy was 468 days, although MST of patients under targeted therapy as the third or fourth line was reported between 6 and 12 months (25-28). MST of patients under PPV alone was 226 days. It is of note that these patients did not tolerate either chemotherapy or targeted therapy, and only best supportive care was applicable for these patients. There are a very few clinical studies for such populations to examine OS, but MST of these patients was reported as <6 months (28). Based on the potential clinical benefits and the safety profile, a next step of clinical trial of PPV with or without chemotherapy or targeted therapy would be warranted in heavily treated advanced NSCLC patients.

Based on the biomarker, antigen-specific CTL response was suggested to be a favorable factor in this study, since MST of patients with (n=11) or without (n=12) CTL responses to the vaccinated peptides in the PPV/chemotherapy group was 692 or 305 days, respectively (p=0.1838). Furthermore, MST with or without CTL responses in the PPV alone group was undefined (n=5) or 210 days (n=13) (p=0.0735), respectively. On the contrary, this might be the opposite in antigen-specific IgG response, since MST of the patients with (n=11) or without (n=9) increased IgG responses in the PPV/chemotherapy group

Table V. Immune responses to peptides in the PPV alone group.

Patient no.	No. of vaccinated peptide[a]	No. of peptides with enhanced IgG responses[b]	No. of peptides with enhanced CTL responses[c]
1	4	0	0
2	4	1	1
3	4	1	0
4	4	NA	NA
5	4	3	1
6	4	2	1
7	4	0	NA
8	4	NA	NA
9	2	0	NA
10	4	1	1
11	4	NA	NA
12	4	0	0
13	4	2	0
14	4	1	1
15	2	0	0
16	3	2	0
17	4	0	0
18	4	NA	NA

PPV, personalized peptide vaccine; NA, not assessed. [a]Peptide numbers used for 1st cycle of vaccination. [b]If the titers of peptide-specific IgG at the end of 1st cycle were >2-fold higher than those in the pre-vaccination plasma, the changes were considered to be enhanced. [c]CTL responses were determined by the number of spots per 10^5 peripheral blood mononuclear cells (PBMCs) reactive with the vaccinated peptides in IFN-γ ELISPOT assay before and after 1st cycle of vaccination.

was 302 or 692 days (p=0.1093), respectively. However, this phenomenon was not observed in patients with PPV alone, since MST of the patients with (n=8) or without (n=5) increased IgG responses in this group was 321 or 226 days (p=0.6305), respectively. We previously reported that peptide-specific IgG response was a favorable factor of OS for hormone refractory prostate cancer or other types of patients under PPV (14,18). PPV in those studies, however, was not combined with chemotherapy. We are now addressing the mechanisms involved in such discrepancy in the peptide-specific IgG responses between the PPV-treated patients with and without combined chemotherapy.

A greater number of peptides showed IgG responses to HLA-matched non-vaccinated peptides after vaccination in the PPV/chemotherapy group as compared to those in the PPV alone group (p=0.004). We previously reported that the epitope spreading assessed by IgG responses to non-vaccinated peptides is a favorable factor for OS of soft-tissue sarcoma patients under PPV (13). Indeed, in the PPV/chemotherapy group, MST of the patients with (n=10) or without (n=10) an increase in IgG responses to non-vaccinated HLA-matched peptides was 692 or 302 days, respectively. Epitope spreading assessed by CTL activity was reported to be associated with clinical responses in some clinical trials (29,30). Chemotherapy-induced tumor cell death could promote antigen presentation by antigen presenting cells to T cells, which might be in part responsible for the epitope spreading in patients in the PPV/chemotherapy group.

By IHC analysis, 12 out of 15 vaccine antigens, from which the vaccine peptides used for PPV were derived, were expressed in primary NSCLC tissues. Lck antigen, a unique vaccine antigen preferentially expressed both in T cells and metastatic tumor cells (21,22), was expressed in a small fraction of tumor cells in some of SCC tissues. None of the remaining three prostate-related antigens (PAP, PSA and PSMA) were detectable in any of these tissues tested. However, at least PSMA and PAP were reported to be expressed in NSCLC cells (31-33), and PSA was also reported to be expressed in certain types of adenocarcinoma cells (34). Therefore, it could not be emphasized strongly at the present time that none of these prostate related antigens were expressed in tumor cells from NSCLC patients, but peptides derived from these three antigens should be removed from the vaccine peptide candidates in the next PPV for NSCLC patients as reported previously (12).

In conclusion, in a third or fourth line treatment of advanced NSCLC, the PPV, compared with chemotherapy, had a possibility of prolongation of OS. Further evaluation of PPV by prospective randomized trials could be recommended for heavily treated advanced NSCLC.

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Personalized peptide vaccination for advanced colorectal cancer

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Keywords: biomarker, colorectal cancer, IL-6, peptide vaccine, personalized vaccine

Abbreviations: aCRC, advanced colorectal cancer; CTL, cytotoxic T lymphocyte; HR, hazard ratio; OS, overall survival; PPV, personalized peptide vaccination.

We have developed a novel approach in cancer immunotherapy, the personalized peptide vaccination (PPV), in which human leukocyte antigen (HLA)-matched peptides are selected on the basis of preexisting host immunity before vaccination. Recently, we demonstrated the feasibility of PPV in previously treated patients with advanced colorectal cancer patients, thus warranting further clinical development of this approach.

Introduction

Recent advances in chemotherapy and/or targeted therapy have improved clinical outcomes in patients with advanced colorectal cancer (aCRC); however, the prognosis remains poor. The development of new therapeutic approaches, including immunotherapy, is thus urgently needed. However, limited numbers of clinical trials of immunotherapies have been reported for patients with aCRC. We have developed a novel approach of cancer immunotherapy, the personalized peptide vaccination (PPV), in which human leukocyte antigen (HLA)-matched peptides are individually selected from a panel of 31 cytotoxic T lymphocyte (CTL) epitope peptides derived from 15 tumor-associated antigens.[1,2] The most unique aspect of PPV is the personalized selection of ideal antigen peptides for individual patients on the basis of both HLA-class I types and preexisting immune responses to peptide vaccine candidates before vaccination.[1,2] In view of the heterogeneity of tumors and the complexity and diversity of immune responses, this approach might be more rational than the selection of non-personalized universal tumor antigens (Fig. 1). As tumor tissues are unavailable and difficult to precisely characterize in most of advanced patients, we selected and administered multiple (up to 4) antigens to increase the possibility that the antigens used for vaccination are expressed in tumor cells.

Early-phase clinical trials of PPV showed the feasibility of this new approach in patients with various types of cancers.[1-4] Recently, we conducted a phase 2 study to examine the feasibility of PPV in previously treated patients with aCRC who had failed at least 1 regimen of standard chemotherapies and/or targeted therapies.[5] Two to 4 HLA-matched peptides were individually selected from a pool of peptide vaccine candidates and administered subcutaneously without severe adverse events, as described previously.[1-4] The median overall survival (OS) time from the first vaccination was 498 d (95% confidence interval [CI], 233–654 days) with 1- and 2-year survival rates of 53% and 22%, respectively. Notably, patients, who had a treatment history of 2 or more regimens of standard chemotherapy and were refractory or intolerant to irinotecan, oxaliplatin, and fluoropyrimidines prior to enrollment showed median OS of 375 d (95% CI, 191–561 days) from the first vaccination, suggesting a potential survival benefit of PPV in previously treated patients with aCRC, even in the refractory stage. Boosting of CTL responses specific to the administered peptides was observed in 63% of patients who completed the first cycle of 6 vaccinations. Importantly, increased peptide-specific CTL responses after vaccination were significantly predictive of favorable OS independently of other factors, suggesting a causal relationship between the biological and clinical efficacies of PPV.

Several post-vaccination biomarkers, such as immune (CTL and/or immunoglobulin G [IgG]) responses to the vaccine antigens, delayed-type hypersensitivity, and autoimmunity, have been reported to be associated with clinical responses in cancer immunotherapies;[1,2,6,7] however, there are currently no validated pre-vaccination predictive biomarkers in widespread use. Not all patients showed clinical benefits from PPV; therefore, we tried to identify prognostic or predictive biomarkers in patients with aCRC who were treated with PPV.[5] By the Cox proportional hazards model, higher interleukin (IL)-6 and interferon gamma-inducible protein-10 (IP-10) and lower B-cell activating factor (BAFF) levels in pre-vaccination plasma were significantly associated with unfavorable OS (hazard ratio [HR] = 1.508, $P = 0.043$; HR = 1.579, $P = 0.024$; HR = 0.509, $P = 0.002$; respectively), although these factors might be not necessarily be predictive and unique to PPV. Notably, however, the

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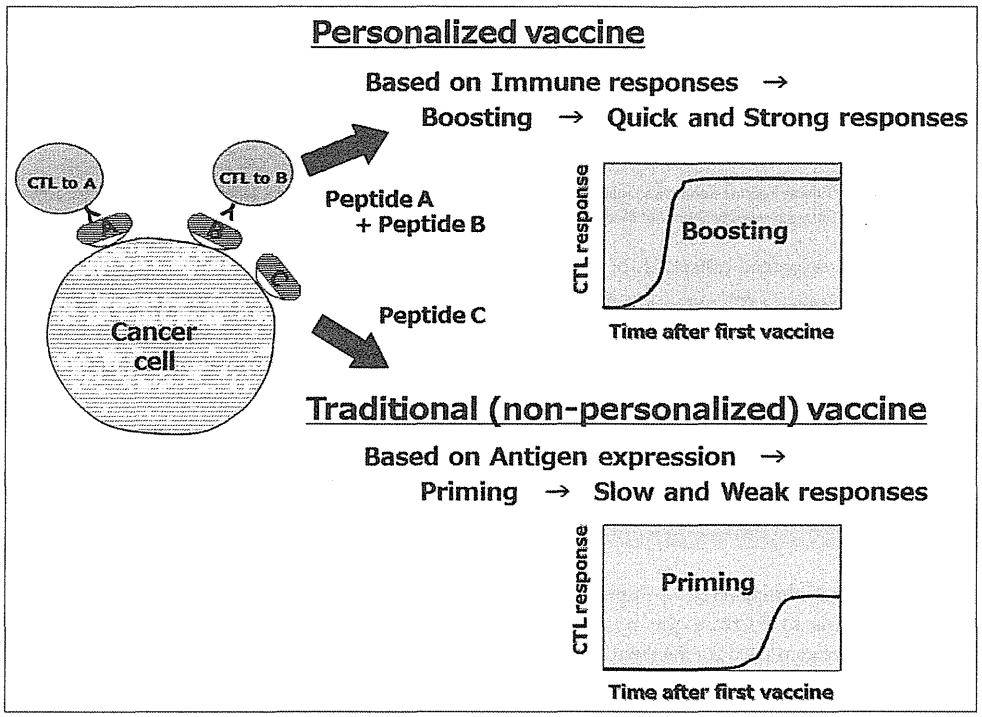

Figure 1. Advantage of personalized peptide vaccine. Personalized vaccine antigens selected on the basis of preexisting host immunity might be better than non-personalized antigens because they can induce quicker and stronger immune responses.

pre-vaccination IP-10 level was predictive of the increase in CTL responses (odds ratio, 0.427; $P = 0.039$), which was associated with improved OS after vaccination, suggesting that IP-10 might potentially be useful for selecting patients with aCRC who would benefit from PPV. To more clearly assess the causal relation of IP-10, CTL responses, and OS as well as to elucidate prognostic versus the predictive relevance of such biomarkers, future randomized, controlled clinical trials with or without PPV would be required.

We have demonstrated that IL-6 might be useful for predicting OS in PPV-treated patients with various types of cancers including aCRC.[5,8,9] As IL-6 has recently been reported to induce suppressive immune cell subsets, such as myeloid-derived suppressor cells and Th17, high levels of IL-6 may inhibit immune responses to cancer vaccines by inducing these suppressive cells. Based on these findings, an early-phase clinical trial is underway to examine whether inhibition 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 patients with aCRC who show higher plasma IL-6 levels. Interestingly, we demonstrated that the IL-6R 48892A>C polymorphism might have a significant effect on OS in patients with aCRC after PPV: the patients bearing the IL-6R 48892C/C or 48892A/C genotypes tended to have a better prognosis than those carrying the IL-6R 48892A/A genotype.[5] As the IL-6R 48892A>C polymorphism has been reported to show no effects on prognosis in patients with some cancers, such as esophageal squamous cell carcinoma and neuroblastoma, without cancer vaccines the prognostic significance of this polymorphism might be unique to vaccinated patients.

In summary, our recently conducted phase 2 trial demonstrated that PPV induced substantial immune responses to vaccine antigens without severe adverse events and showed a potential clinical benefit in previously treated patients with aCRC even in the refractory stage.[5] Nevertheless, because it was a small study with a limited number of patients, some of whom received combined chemotherapies and/or targeted therapies during the vaccination period, the clinical efficacy of PPV, as well as the clinical utility of the identified biomarkers, in patients with aCRC remain to be confirmed in future, larger scale, randomized trials of PPV without combined chemotherapies or targeted therapies.

Disclosure of Potential Conflicts of Interest

Kyogo Itoh owns stock in the Green Peptide Co., Ltd., and has received research funding from Taiho Pharmaceutical Co., Ltd. No potential conflicts of interests were declared by the other authors.

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Serum Tri- and Tetra-Antennary *N*-Glycan is a Potential Predictive Biomarker for Castration-Resistant Prostate Cancer

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BACKGROUND. The U.S. FDA has approved several novel systemic agents including abiraterone acetate and taxoid cabazitaxel for metastatic castration-resistant prostate cancer (CRPC) result in a complicated decision-making while selecting an appropriate treatment. Therefore, a predictive biomarker for CRPC would provide useful information to physicians. The aim of this study is to evaluate the diagnostic potential of serum *N*-glycan profiling in CRPC.

METHODS. Serum *N*-glycomics was performed in 80 healthy volunteers and 286 benign prostatic hyperplasia, 258 early-stage PC, 46 PC with androgen deprivation therapy (ADT), and 68 CRPC patients using the glycoblotting method. A total of 36 types of *N*-glycan levels in each patient were analyzed using logistic regression analysis and receiver operating characteristic curves. We also examined the expression of *N*-glycan branching enzyme genes in PC cell lines using quantitative RT-PCR.

RESULTS. We observed that tri- and tetra-antennary *N*-glycans were significantly higher in CRPC patients than in any other groups. The longitudinal follow-up of tri- and tetra-antennary *N*-glycan levels revealed that one PC with ADT patient showed an increase that was more than the cut-off level and two consecutive increases in tri- and tetra-antennary *N*-glycan levels 3 months apart; resulted in biochemical recurrence despite the castrate level of testosterone, and the patient was defined as CRPC. Expression of *N*-glycan branching enzyme genes were significantly upregulated in CRPC cell lines.

CONCLUSIONS. These results suggest that the overexpression of tri- and tetra-antennary *N*-glycan may be associated with the castration-resistant status in PC and may be a potential predictive biomarker for CRPC. *Prostate 74:1521–1529, 2014.* © 2014 Wiley Periodicals, Inc.

KEY WORDS: **serum *N*-glycan; androgen deprivation therapy; biomarker; castration-resistant prostate cancer; glycoblotting**

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INTRODUCTION

Prostate cancer (PC) is one of the most common cancers in men worldwide [1]. The American Cancer Society estimated 241,740 new cases and 28,170 deaths in the United States in 2012 [2]. PC is a multifocal disease with a moderate clinical progression. Localized early-stage PC (esPC) can be well treated with radical prostatectomy. In contrast, advanced PC is mostly treated with androgen deprivation therapy (ADT); however, ADT fails in approximately 10%–20% of patients, who then develop castration-resistant PC (CRPC) within 5 years of follow-up [3,4]. CRPC is a heterogeneous and progressive stage of PC and includes both symptomatic and asymptomatic male patients with or without clinical metastases [5]. Although the mechanism underlying androgen independence remains unclear, recent advances have led to a better understanding of this mechanism. Over the past few years, several novel systemic agents for metastatic CRPC, such as the androgen synthesis inhibitor abiraterone acetate [6], the immunotherapeutic sipuleucel-T [7], the taxoid cabazitaxel [8], and the enzalutamide [9], have been approved by the US Food and Drug Administration (FDA). Therapeutic option for CRPC becomes complicated treatment decision making. Therefore, a predictive biomarker for CRPC would provide useful information to physicians for selecting the appropriate therapy sequence at a given time as soon as possible. However, no validated predictive biomarkers for CRPC have been reported.

Glycosylation plays an important role in various biological functions. Cancer-associated aberrant glycosylation has been frequently observed in bladder cancer [10], , germ cell tumors [11], PC [12], colorectal cancer [13], hepatocellular cancer [14], pancreatic cancer [15], and renal cell carcinoma [16]. Recently, high-throughput, comprehensive, and quantitative *N*-glycomics based on the glycoblotting method using Sweetblot revealed that serum *N*-glycomics is promising to screen for a diagnostic and prognostic marker for renal cell carcinoma [17]. It is also a promising prognostic tool in patients undergoing hemodialysis [18] and patients with advanced hepatocellular carcinoma undergoing treatment with sorafenib [19]. However, the use of serum *N*-glycans as a predictive biomarker for PC has not yet been investigated. In the present study, we performed serum *N*-glycomics in PC patients and evaluated its potential as a predictive biomarker for CRPC.

MATERIALS AND METHODS

Serum Samples

A total of 650 patients with benign prostatic hyperplasia (BPH), esPC, PC with ADT, or CRPC were treated at our hospital between June 2007 and December 2013. Serum samples from BPH (n = 286) and esPC (n = 258) patients were obtained at the time of biopsy. The final diagnosis of BPH and esPC patients was confirmed using the histopathological findings of prostate biopsies. Serum samples from PC with ADT (n = 46) and CRPC (n = 68) patients were obtained at the time of treatment. Biochemical recurrence was defined as prostate-specific antigen (PSA) levels >0.2 ng/ml after prostatectomy or increase 2 ng/ml above the nadir PSA after radiotherapy (RT). CRPC was defined by PSA or radiographic progression despite the castrate levels of testosterone of <50 ng/dl. All samples were stored at −80°C until use. Serum samples from 80 healthy volunteers (HLT) were obtained from our serum bank and were stored at −80°C until use. The study was performed in accordance with the ethical standards of the Declaration of Helsinki and was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine. Informed consent was obtained from all patients. Patient demographics are shown in Table I.

Glycoblotting Method and Mass Spectrometry

Serum *N*-glycan analysis was performed as described previously using SweetBlot™ (System Instruments, Hachijo, Japan) [17] (Supplementary Fig. 1). Briefly, 10 μl of serum samples containing 40 pmol of the internal standard disialo-galactosylated biantennary *N*-glycan, which has amidated sialic acids (A2 amide glycans) (Supplementary Table I), were reduced and alkylated using DTT and iodoacetamide (Wako Pure Chemical Industries, Osaka, Japan), respectively. The resulting mixture was then trypsinized and heat inactivated. After cooling down to room temperature, peptide *N*-glycanase F (New England BioLabs, Ipswich, MA) was added to the mixture to release total serum *N*-glycans. After incubating for 360 min at 37°C, 20 μl of the resulting mixture was equivalent to 2.5 μl of serum. An aliquot of each pretreated sample was mixed with 500 μl of BlotGlyco H beads (Sumitomo Bakelite, Co., Tokyo, Japan) to capture glycans via stable hydrazone bonds on MultiScreen Solvinert® filter plate (MerkMillipore, Billerica, MA). Then, acetyl capping of unreacted hydrazide functional groups on the beads and methyl esterification of sialic acid carboxyl groups, which exist in the terminal of the captured glycans, were performed sequentially; serial washes were then performed before each step, as described previously [17,19,20–24]. The captured *N*-glycans were labeled with benzyloxiamine (BOA, Sigma-Aldrich, St. Louis, MO) by transiminization and were eluted in 150 μl of water. The BOA-labeled

TABLE I. Patient Demographics of the Study Cohort

	HLT	BPH	esPC	PC with ADT	CRPC
Patients (*n*)	80	286	258	46	68
Age,mean ± SD	64 ± 13	67 ± 8	68 ± 7	77 ± 7	74 ± 7
No. of males/females	47/33	286/0	258/0	46/0	68/0
Median iPSA (range)		6.4 (0.6–19.7)	7.4 (2.2–17.9)	23.0 (5.5–4564)	127 (1.3–17340)
Median nPSA (range)				0.08 (0–2.6)	
Median ADT follow-up (months)				42.5	
Bone metastasis, *n* (%)				4 (8.7)	53 (77.9)
BCR, *n* (%)				19 (41.3)	68 (100)

Ipsa, initial PSA value at diagnosis; nPSA, nadir PSA; BCR, biochemical recurrence.

glycans were detected using MALDI-TOF MS (Ultraflex 3 TOF/TOF mass spectrometer, Bruker Daltonics, Bremen, Germany). Compositions and structures of glycans were predicted using GlycoMod Tool (http://br.expasy.org/tools/glcomod).

Quantitative Reproducibility Test of Sweetblot

Each quantitative reproducibility test of Sweetblot was performed as described previously [25]. Briefly, serum samples and serially diluted standard human serum (Sigma-Aldrich) were added to the plate, and the whole process of *N*-glycomics was performed with Sweetblot. The peak area of each glycan detected at 0.5×, 0.75×, 1×, 1.25×, 1.5×, 1.75×, 2×, and 2.25× concentrations was plotted. This assay was repeated twice, and quantitative reliability was then judged based on following parameters: outliers were allowed <3 points, slope σ of <3.0, and the significance level of the correlation coefficient r was <0.05. Glycan peaks were judged to be useful when the above mentioned criteria of the assay were met, and the resulting glycans were used for statistical analysis.

Statistical Analysis

Statistical calculations for clinical data were performed using SPSS verion 20.0 (SPSS, Inc., Chicago, IL) and GraphPad Prism 6.03 (GraphPad Software, San Diego, CA). Intergroup differences were statistically compared using the Student's *t*-test for normally distributed models or the Mann–Whitney U-test for nonnormally distributed models. *N*-glycan levels were analyzed using logistic regression analysis and receiver operating characteristic (ROC) curves to select *N*-glycans that were associated with CRPC status in PC. The optimal cut-off points were calculated using the following formula: $(1 - \text{sensitivity})^2 + (1 - \text{specificity})^2$ [26]. $P < 0.05$ was considered significant.

Real-time Quantitative RT-PCR

The normal prostate epithelial cell line RWPE-1 and the PC cell lines LNCaP, DU145, and PC-3 were obtained from the American Type Culture Collection. RWPE-1 was grown at 37°C with 5% CO_2 in Keratinocyte-SFM medium supplemented with penicillin, streptomycin, bovine pituitary extract, 5 ng/ml epidermal growth factor. LNCaP, DU145, and PC-3 were grown at 37°C with 5% CO_2 in RPMI-1640 medium supplemented with penicillin, streptomycin, and 10% FBS. LNCaP-androgen independent (AI) cell were grown at 37°C with 5% CO_2 in RPMI-1640 medium supplemented with penicillin, streptomycin, and 10% charcoal-stripped FBS. Total RNA was isolated from RWPE-1, LNCaP, LNCaP-AI, DU145, and PC-3 cells using ISOGEN II (Wako Pure Chemical Industries) according to the manufacturer's instructions. First-strand cDNA was synthesized from 0.5 μg of total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Kita-ku, Osaka, Japan) according to the manufacturer's instructions. Real-time qRT-PCR assays were performed in triplicate using GeneAce SYBR® qPCR Mix α No ROX (Nippon Gene, Chiyoda-ku, Tokyo, Japan) and 500 nM gene-specific primers. Reactions were processed on a CFX connect™ Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA) under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 45 sec. PrimeTime® qPCR primer pairs for human *N*-acetylglucosaminyltransferase I (*MGAT1*) (Hs.PT.58.4702749), human *N*-acetylglucosaminyltransferase II (*MGAT2*) (Hs.PT.58.24612062.g), human *N*-acetylglucosaminyltransferase III (*MGAT3*) (Hs.PT.58.26307986.g), human *N*-acetylglucosaminyltransferase IVa (*MGAT4A*) (Hs.PT.58.3289156), human *N*-acetylglucosaminyltransferase IVb (*MGAT4B*) (Hs.PT.58.19371732), human *N*-acetylglucosaminyltransferase IVc (*MGAT4C*) (Hs.PT.58.2945729), human

N-acetylglucosaminyltransferase V (*MGAT5A*) (Hs.PT.58.4758371), human *N*-acetylglucosaminyltransferase Vb (*MGAT5B*) (Hs.PT.58.27758528), and human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Hs.PT.39a.22214847) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Relative expression levels of *MGAT* genes were normalized to expression of the *GAPDH* gene.

RESULTS

Tri- and Tetra-Antennary *N*-glycans Significantly Increased in CRPC Patients

Serum *N*-glycan analysis performed using the glycoblotting method and mass spectrometry identified 45 types of BOA-labeled *N*-glycans in all serum samples. We then performed quantitative reproducibility tests. Finally, 36 types of *N*-glycans (Supplementary Table I) had good quantitative reproducibility among all samples and could be used for statistical analysis. Table I summarizes the demographics of the study cohort. No significant differences were observed in age between BPH and esPC groups. The iPSA level in the esPC group was significantly higher than that in the BPH group ($P = 0.0002$). The age of patients in the PC with ADT group was significantly higher than that in the CRPC group ($P = 0.033$). No significant differences were observed in the *N*-glycan profiles of HLT, BPH, esPC, and PC with ADT patients. We observed significant differences in the *N*-glycan profiles between CRPC and the other groups. Nine *N*-glycans (*m/z* 1362, 1566, 1753, 1794, 3049, 3414, 3560, 3719, and 3865) were significantly different between PC with ADT and CRPC groups (Table II, Supplementary Figs. 2 and 3). To investigate predictive potential for CRPC, nine *N*-glycans were analyzed using logistic regression analysis. The tri- and tetra-antennary *N*-glycans *m/z* 3049 (odds ratio, 3.326) and *m/z* 3414 (odds ratio, 13.189) showed higher odds ratio than other glycans, therefore

TABLE III. Logistic Regression Analysis of Serum *N*-glycans for Prediction of CRPC

m/z	Coefficient	Odds ratio	Odds ratio (95%CI)	*P*-value
1362	−0.476	0.621	0.313–1.234	0.174
1566	0.108	1.114	0.839–1.479	0.457
1753	−0.644	0.525	0.280–0.983	0.044
1794	0.265	1.304	1.002–1.697	0.048
3049	1.202	3.326	1.199–9.226	0.021
3414	2.579	13.189	3.477–50.030	<0.0001
3560	−0.189	0.828	0.465–1.475	0.521
3719	−1.535	0.215	0.073–0.633	0.005
3865	0.622	1.863	1.072–3.238	0.027

m/z 3049 and *m/z* 3414 were selected as specific *N*-glycans for the prediction of CRPC (Table III). Figure 1A and B showed serum level of *m/z* 3049 and *m/z* 3414 glycans in each group. ROC curves were then used to compare the predictive potential of *m/z* 3049 and *m/z* 3414 for CRPC (Fig. 1C). The area under the curve (AUC) of *m/z* 3049 and *m/z* 3414 could be used to discriminate between PC with ADT and CRPC patients (AUC, 0.697 and 0.748, respectively).

Longitudinal Follow-up of Tri- and Tetra-antennary *N*-glycan Levels in 16 PC With ADT Patients

The optimal cut-off levels of *m/z* 3049 and *m/z* 3414 were determined to be >1.60 μM and >1.36 μM, respectively, for the prediction of CRPC based on ROC curves (Table IV). To evaluate the predictive potential of *m/z* 3049 and *m/z* 3414, we followed-up *m/z* 3049 and *m/z* 3414 levels in 16 PC with ADT patients every 3 or 6 months (Fig. 2A and B). Total PSA and testosterone levels were also followed-up at the same time points (Figs. 2C and D). We found that one PC

TABLE II. Results of Serum *N*-glycomics Were Significantly Different Between PC With ADTand CRPC Patients

	Mean ± SD level (μM)			ROC curve
m/z	PC with ADT	CRPC	*P*-value	AUC (95%CI)
1362	1.79 ± 0.47	1.59 ± 0.41	0.042	0.612 (0.508–0.717)
1566	2.50 ± 1.22	2.21 ± 1.22	0.047	0.609 (0.503–0.716)
1753	2.23 ± 0.62	1.89 ± 0.73	0.022	0.626 (0.526–0.728)
1794	3.87 ± 2.46	2.95 ± 1.72	0.042	0.612 (0.508–0.716)
3049	1.54 ± 0.52	2.09 ± 0.91	0.0003	0.697 (0.599–0.794)
3414	1.27 ± 0.32	2.01 ± 0.99	<0.0001	0.748 (0.659–0.837)
3560	1.28 ± 0.99	1.98 ± 1.91	0.033	0.617 (0.515–0.720)
3719	1.14 ± 0.30	1.89 ± 0.95	<0.0001	0.753 (0.666–0.810)
3865	1.19 ± 0.78	1.89 ± 2.10	0.014	0.636 (0.534–0.738)

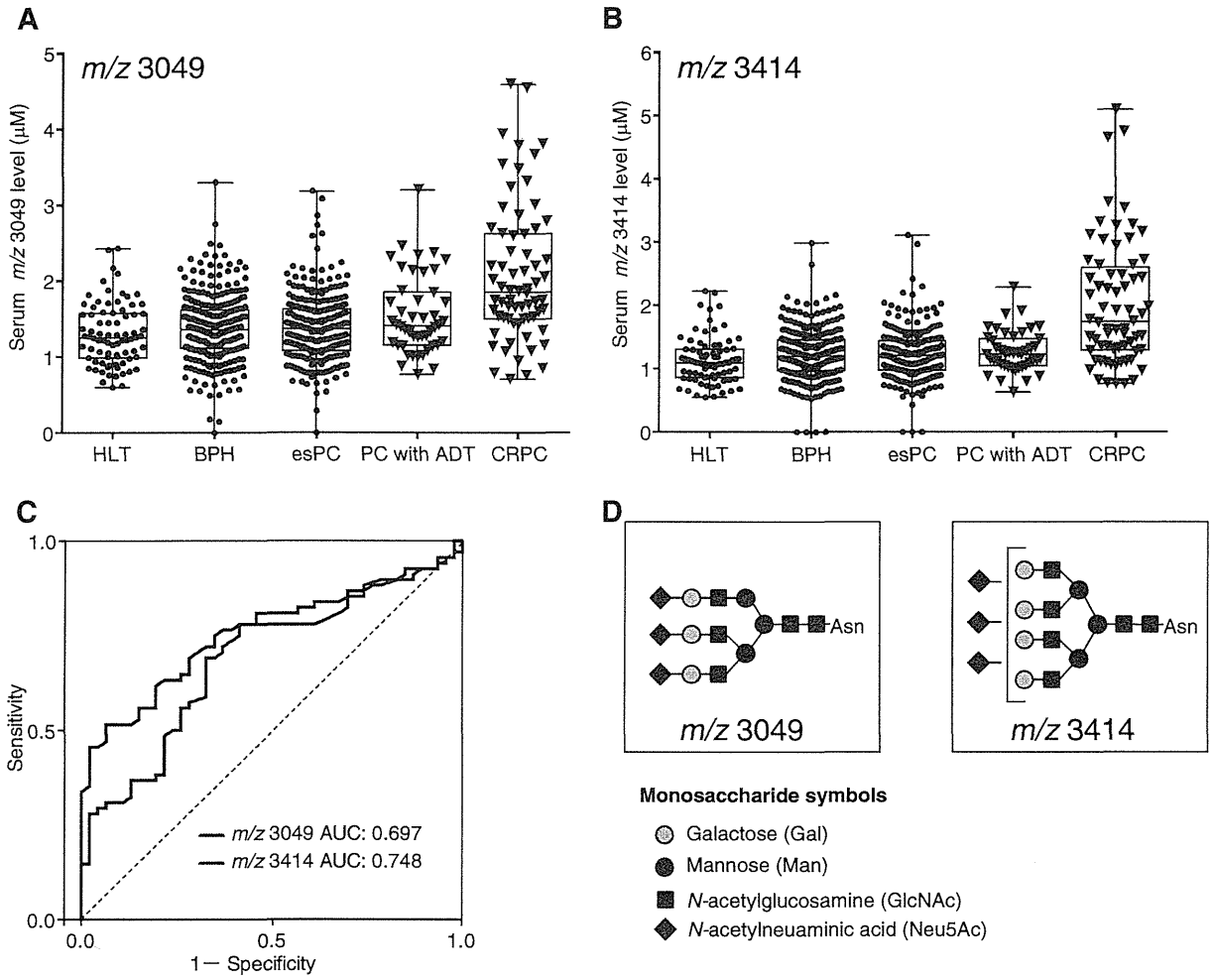

Fig. 1. Serum levels of significant tri- and tetra-antennary *N*-glycans associated with the prediction of CRPC that were selected using logistic regression analysis. (A) Serum *m/z* 3049 level in HLT, BPH, esPC, PC with ADT, and CRPC patients. (B) Serum *m/z* 3414 level in HLT, BPH, esPC, PC with ADT, and CRPC patients. (C) Receiver operating characteristics (ROC) curve for the prediction of CRPC. The AUCs of *m/z* 3049 and *m/z* 3414 were 0.697 and 0.748, respectively. (D) Putative structures of *m/z* 3049 and *m/z* 3414 are represented as monosaccharide symbols. Yellow circles, galactose (Gal); green circles, mannose (Man); blue squares, *N*-acetylgulucosamine (GlcNAc); purple diamonds, *N*-acetylneuraminic acid (Neu5Ac).

with ADT patient showed two consecutive increases in *m/z* 3049 and *m/z* 3414 levels 3 months apart. This patient also showed two consecutive increases in PSA levels and was finally defined as CRPC because the testosterone level was <50 ng/dl. This finding suggests that the overexpression of serum tri- and tetra-antennary *N*-glycans may be associated with the castration-resistant status in PC.

Transcription Levels of *N*-glycan Branching Enzyme Genes Were Significantly Upregulated in CRPC Cell Lines

We also examined transcription levels of *MGAT1*, *MGAT2*, *MGAT3*, *MGAT4A*, *MGAT4B*, *MGAT5A*, and *MGAT5B*, which are medial Golgi enzymes that initiate the β1,6GlcNAc branching in bi-, tri-, and tetra-branched *N*-glycans, in PC cell lines using qRT-PCR (Fig. 3). The CRPC-like cell lines DU145 and PC-3 showed significantly increased transcription of *MGAT1*, *MGAT2*, *MGAT4B*, *MGAT5A*, and *MGAT5B* genes. Particularly, the expression of the *MGAT5B* gene was 20-fold higher in CRPC like LNCaP-AI, DU145, and PC-3 cells than in androgen-dependent LNCaP cells and normal prostate epithelial RWPE-1 cells.

DISCUSSION

High-throughput, comprehensive, and quantitative *N*-glycomics is an important and promising method.

TABLE IV. Optimal Cut-off Levels of *m/z* 3049 and *m/z* 3414, Sensitivity, Specificity, Accuracy, and Predictive Value

m/z	Cut-off (μM)	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)
3049	>1.595	69.1	37.0	56.1	61.8	44.7
3414	>1.355	69.1	41.3	57.9	63.5	47.5

PPV, positive predictive value; NPV, negative predictive value.

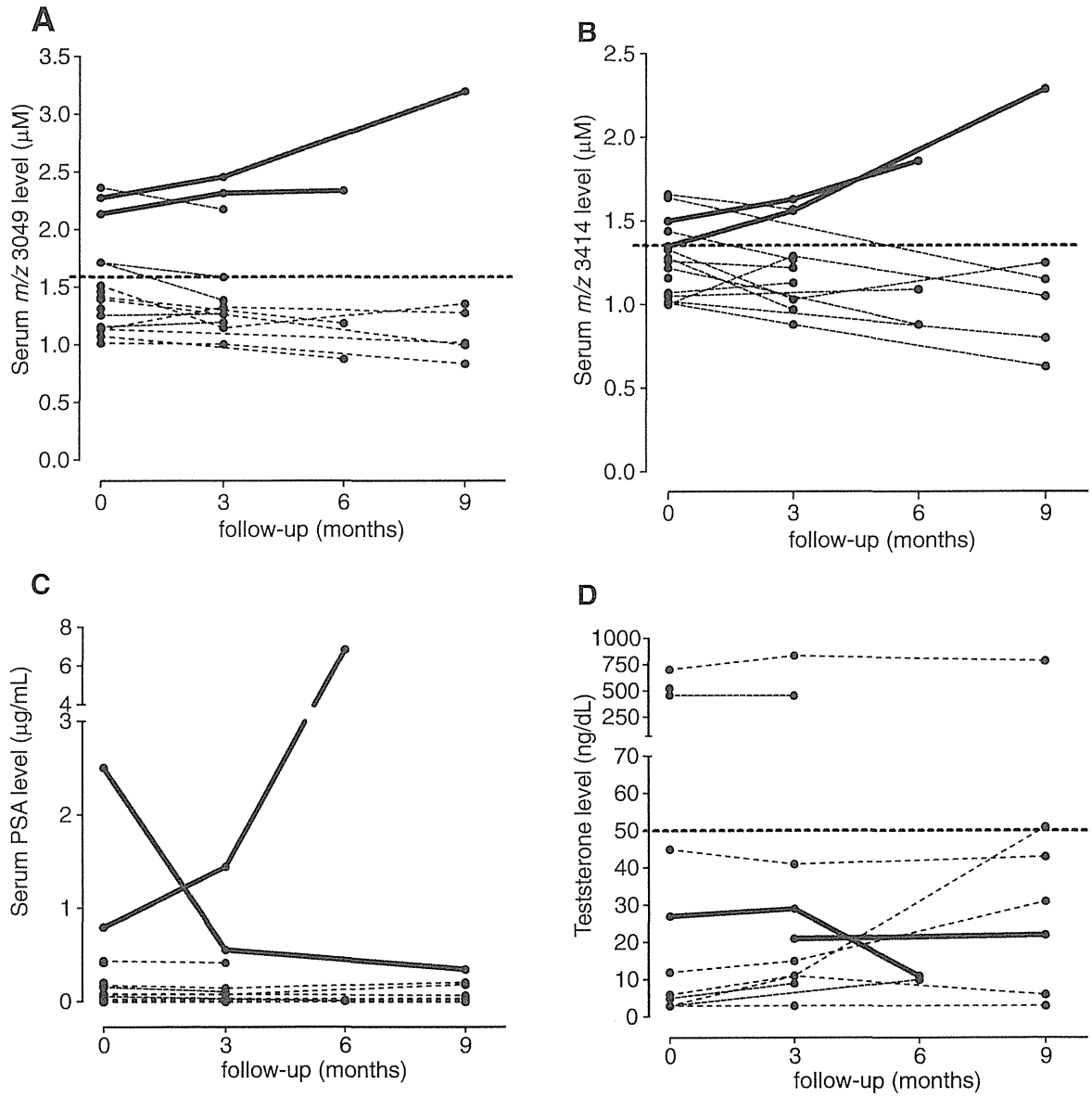

Fig. 2. The longitudinal follow-up of serum *m/z* 3049, *m/z* 3414, PSA, and testosterone levels in PC with ADT patients. (A) Serum *m/z* 3049 levels. The red dashed line represents the optimal cut-off level of *m/z* 3049 (>1.60 μM). (B) serum *m/z* 3414 levels. The red dashed line represents the optimal cut-off level of *m/z* 3414 (>1.36 μM). (C) Total serum PSA levels. (D) Serum testosterone levels. The red dashed line represents the castrate level of testosterone (50 ng/dL). Blue and pink bold lines in panels A and B indicate the PC patient who was treated with ADT and then experienced two consecutive increases in tri- and tetra-antennary *N*-glycan levels. Only the blue bold line shows the PC with ADT patient who experienced two consecutive increases in PSA levels (panel C) despite maintaining a castrate level of testosterone (panel D); he was finally defined as CRPC.

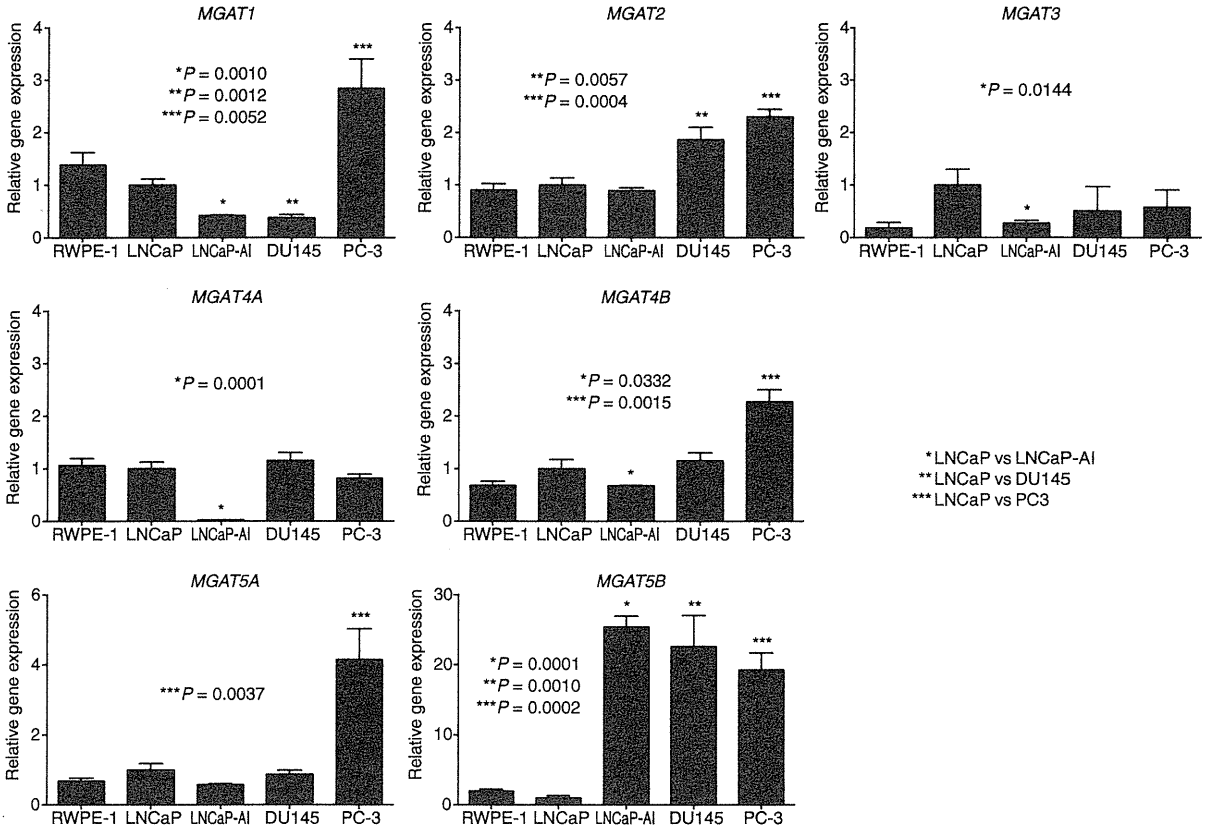

Fig. 3. Quantitative qRT-PCR of *N*-glycan branching enzymes (*MGATs*) in PC cell lines. Relative expression levels of *MGAT* genes were normalized to the expression of the *GAPDH* gene in each cell line. The expression of each *MGAT* gene in LNCaP cells was used as control and was defined as 1.0. Asterisk symbol indicate *P* value of LNCaP versus LNCaP-AI. Double asterisk symbol indicate *P* value of LNCaP versus DU145. Triple asterisk symbol indicate *P* value of LNCaP versus PC-3.

Several studies have reported that differences in glycan profiling between diseased and benign states may be useful in the diagnosis or prognosis of diseases [17–19,23–25]. In prostate cancer, Kyselova et al. [27] investigated that *N*-glycomic profiles (50 types of *N*-glycan) derived from human blood sera of 10 healthy males were compared to those from 24 metastatic PC patients. Although the sample size was very small, they report tri- and tetra-antennary *N*-glycans of metastatic PC patients were significantly higher than those of healthy males. This was consistent with our present result. In the present study, the recently established technology of *N*-glycan analysis with the glycoblotting method and MALDI-TOF was used for high-throughput, comprehensive, and quantitative serum *N*-glycan profiling in PC patients. To the best of our knowledge, this is the first report to identify serum *N*-glycans as biomarkers in CRPC patients by using high-throughput quantitative *N*-glycomics. Our results demonstrate that serum levels of tri- and tetra-antennary *N*-glycans (*m/z* 3049 and *m/z* 3414) were statistically and significantly different between PC with ADT and CRPC patients using the optimal cut-off points (Figs. 1 and 2). A previous study reported that cancer-associated aberrant glycosylation increases the transcription of the *MGAT5* gene, which initiates β1,6GlcNAc branching in tri- and tetra-branched *N*-glycans in PC and plays an important role in metastasis of PC [28]. Zavareh et al. [29] reported that the knockdown of *N*-acetylglucosaminyltransferase I, which is encoded by the *MGAT1* gene and is the first branching enzyme required for additional branching on *N*-glycan, decreased levels of branched *N*-glycan on the surface of PC-3 cells. In addition, their orthotopic xenograft model exhibited significantly decreased primary tumor growth and incidence of lung metastasis. In the current study, we demonstrated that transcription levels of *MGAT1*, *MGAT2*, *MGAT4B*, *MGAT5A*, and *MGAT5B* genes were significantly upregulated in CRPC cell lines (Fig. 3).

Results of several reports and the current study indicated that the overexpression of tri- and tetra-branched *N*-glycans on the surface of CRPC cells due to upregulation of *N*-glycan branching enzymes (*MGATs*) was strongly correlated with metastatic PC, and this overexpression may be associated with the castration-resistant status in PC.

These results suggest that the use of the glycoblotting method may provide insight into new factors predicting CRPC. Although serum tri- and tetra-antennary *N*-glycan expression was revealed as a useful predictive biomarker in CRPC patients in the current study, this study has several limitations. First, this study is small and preliminary. Second, it is very important to determine the carrier protein for tri- and tetra-antennary *N*-glycans that enables it to be released into the circulation from tumor tissues or circulating tumor cells. Otherwise, the altered serum *N*-glycan profile could be a systematic immunogenic reaction of the released tumor-associated antigen. Future studies should address whether these alterations are a direct result of the castration-resistant status in PC. Third, longitudinal patterns of changes in tri- and tetra-antennary *N*-glycan from PC with ADT to CRPC patients were investigated in only 16 patients. To validate these predictive biomarkers for CRPC, an increased number of patients is required. Despite these limitations, the overexpression of tri- and tetra-antennary *N*-glycans was clearly demonstrated to be a potential biomarker for the prediction of CRPC in this study. Future large-scale prospective validation studies may determine the clinical significance of these carbohydrate biomarkers.

CONCLUSIONS

Although the present study is small and preliminary, quantitative whole serum *N*-glycan profiling may have the potential to predict castration-resistant status in PC. Glycoblotting with MALDI-TOF mass spectrometry may be a promising method for screening of new predictive biomarkers. At present, no validated predictive biomarkers for CRPC have been reported. Therefore, a predictive biomarker for CRPC would provide useful information to physicians to decide the appropriate therapy sequence. Further clinical trials are warranted to investigate the clinical significance of novel carbohydrate markers.

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