Serum tri- and tetra-antennary N-glycan is a potential predictive biomarker for castration-resistant prostate cancer

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John T. Isaacs
Editor-in-Chief
The Prostate

Dear Dr. Isaacs,

Thank you very much for your letter suggesting the revision of our manuscript. The critiques are generally positive and useful and we have revised the manuscript accordingly as follows:

Comments: □□Reviewer 1□
Comments to the Author□
This paper present important data for diagnosis of prostate cancer. The authors uses patient serum and analyzed N-glycan structures using sophisticated methods. However the study has some weakness, which needs to be addressed by revision.

1. It is well-written that malignant cancer cells produce triantenary and tetraantenary glycans. This work is therefore consistent with the previous reports. This should be stated in the manuscript.

[Response]
We added new reference #27 and added following sentence in page17;
In prostate cancer, Kyselova Z 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.

2. Structure of N-glycans were identified by mass spectrometry, but no mass spectrometry data are presented. The authors should present representative mass data each for HLT, BPH, esPC, PC with ADT, and CRPC, as supplemental figures.

[Response]

We added supplemental figure 2 and 3 including representative mass spectra data each for HLT, BPH, esPC, PC with ADT, and CRPC.

We added 5 words in page 4 as follows;

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 2, Supplementary Figs 2 and 3).

We also added supplemental figs 2 and 3 legends in supplementary figure legend section as follows;

Supplementary Figure 2. Representative MALDI-TOF MS spectra (range of m/z 1000 to 4000) of BOA-labeled *N*-glycans derived from HLT, BPH, esPC, PC with ADT and CRPC patient serum. Significantly different *N*-glycans (*m/z* 1362, 1566, 1753, 1794, 3049, 3414, 3560, 3719, and 3865) between CRPC and other groups were shown in mass spectra. Symbols: yellow circles, galactose (Gal); green circles, mannose (Man); blue squares, *N*-acetylgulucosamine (GlcNAc); purple diamonds, *N*-acetylneuraminic acid (Neu5Ac).

Supplementary Figure 3. Representative MALDI-TOF MS spectra (range of m/z 2500 to 4000) of BOA-labeled *N*-glycans derived from HLT, BPH, esPC, PC with ADT and CRPC patient serum. Significantly different *N*-glycans (*m/z* 3049, 3414,

3560, 3719, and 3865) between CRPC and other groups were shown in mass spectra. Symbols: yellow circles, galactose (Gal); green circles, mannose (Man); blue squares, *N*-acetylgulucosamine (GlcNAc); purple diamonds, *N*-acetylneuraminic acid (Neu5Ac).

3. Manuscript need to be edited extensively. It is difficult to understand legend to figure 1.

[Response]

We deeply apologize for our mistake in Figure 1. Putative *N*-glycan structure of *m/z* 3414 (sialyl or Lactosaminyl tri-antennary *N*-glycan) upper left corner of graph B was wrong. Correct putative *N*-glycan structure of m/z *3414* was three terminal sialic acid attached tetra-antennary *N*-glycan ((Hex)4(HexNAc)4(NeuAc)3 + (Man)3(GlcNAc)2)) shown in Supplementary Table 1. Therefore, we revised correct *N*-glycan structure of *m/z* 3414 was shown in Figure 1, panel D. Putative structure of *m/z* 3049 was also transfer in panel D.

In according to above revision, we revised in title and manuscript as follows;

Previous title

Serum sialyl tri-antennary *N*-glycan is a potential predictive biomarker for castration-resistant prostate cancer.

Revised title

Serum tri-and tetra- antennary *N*-glycan is a potential predictive biomarker for castration-resistant prostate cancer.

In manuscript and legend, “sialyl tri-antennary” was instead of “tri- and tetra-antennary”.

To explain detail of fig.1, we added a few sentence in manuscript (page14) as follows;

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 *m/z* 3049 and *m/z* 3414 were selected as specific *N*-glycans for the prediction of CRPC (Table 3). Fig.1A and B showed serum level of *m/z* 3049 and *m/z* 3414 glycans in each group.

We also revise Figure 1 legend as follows;

Figure 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).

By taking into consideration the critiques of the reviewers, we believe that the manuscript has improved, and we hope that it is now acceptable for publication in The Prostate. We thank you very much for your editorial efforts.

Sincerely yours,

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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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Running title: Serum *N*-glycan profiling in CRPC

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Conflicts of interests: All authors declare no conflicts of interests.

Word count: Abstract: 250/250 words, main text: 3813 words, total: 4269words.

Total number of figures and tables: 3 figures and 4 tables, 1 supplementary figure, and 1 supplementary table.

ABSTRACT

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.

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

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 become

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), early-stage PC (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 1.

Glycoblotting Method and Mass Spectrometry

Serum *N*-glycan analysis was performed as described previously using SweetBlot™ (System Instruments, Hachijo, Japan) [17] (Supplementary Figure 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 1), 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, USA) 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, 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, USA). 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, USA) by transiminization and were eluted in 150 μL of water. The BOA-labeled 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 0.5x, 0.75x, 1x, 1.25x, 1.5x, 1.75x, 2x, and 2.25x 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 abovementioned 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 ver. 20.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6.03 (GraphPad Software, San Diego, CA, USA). 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, USA) under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. 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 (*G□□□□*) (Hs.PT.39a.22214847) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). 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 1) had good quantitative

reproducibility among all samples and could be used for statistical analysis. Table 1 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 2, 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 *m/z* 3049 and *m/z* 3414 were selected as specific *N*-glycans for the prediction of CRPC (Table 3). Fig.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 4). 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, B). Total PSA and testosterone levels were also followed-up at the same time points (Fig. 2C, D). We found that one PC 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. 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 Z 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.

ACKNOWLEDGMENTS

The authors thank Dr. Kazuyuki Mori and Mrs. Yukie Nishizawa for their invaluable help with sample collection and patient data management.

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FIGURE LEGENDS

Figure 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).

Figure 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.

Figure 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 vs LNCaP-AI. Double asterisk symbol indicate *P* value of LNCaP vs DU145. Triple asterisk symbol indicate *P* value of LNCaP vs PC-3.

Figure 1

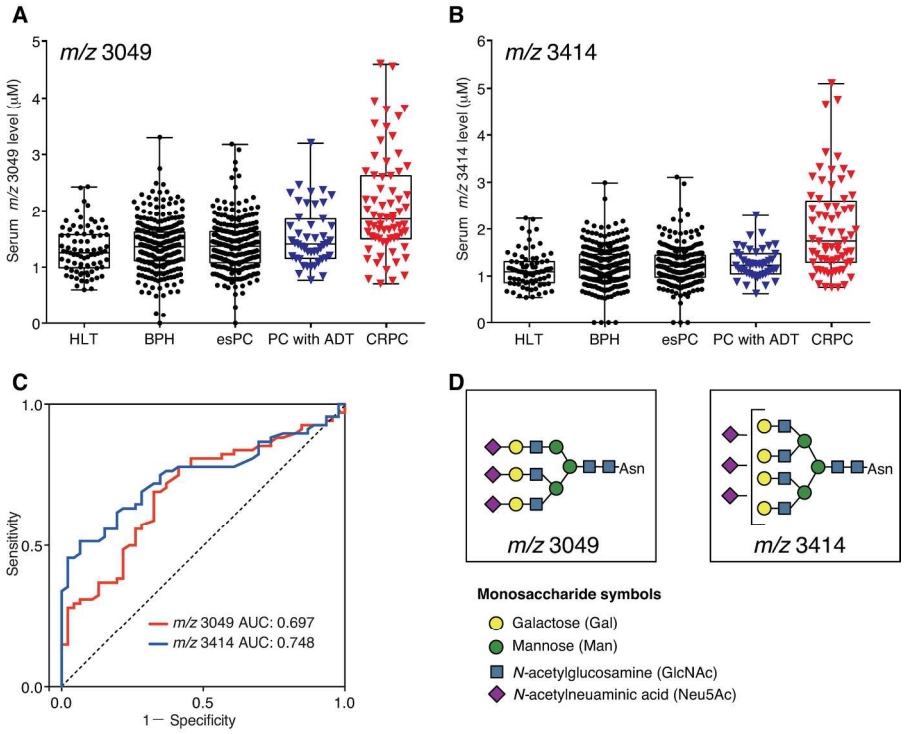

Figure 1. Serum levels of significant tri- and tetra-antennary N-glycans associated with the prediction of CRPC that were selected using logistic regression analysis. A, serum m/z 3049 level in HLT, BPH, esPC, PC with ADT, and CRPC patients. B, serum m/z 3414 level in HLT, BPH, esPC, PC with ADT, and 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).
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Figure 2

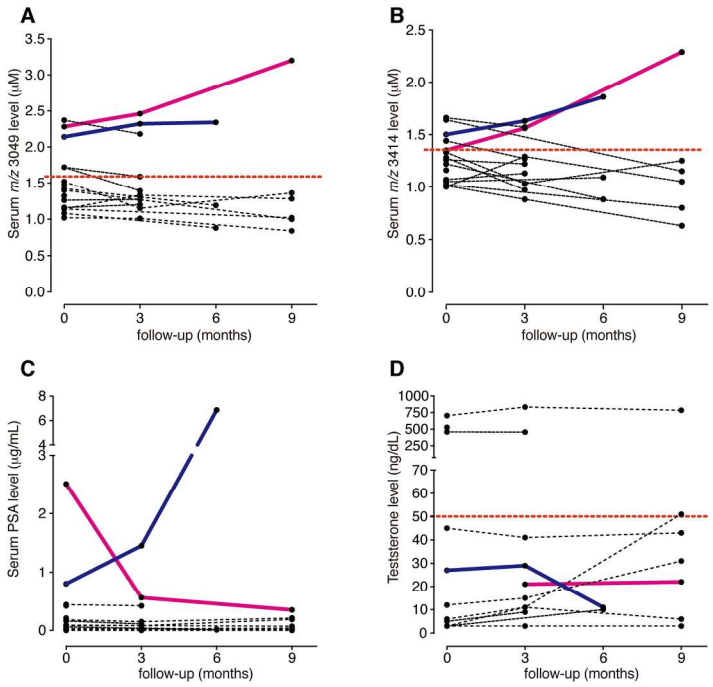

Figure 2. The longitudinal follow-up of serum m/z 3049, m/z 3414, PSA, and testosterone levels in PC with ADT patients. A, serum m/z 3049 levels. The red dashed line represents the optimal cut-off level of m/z 3049 (>1.60 μM). B, serum m/z 3414 levels. The red dashed line represents the optimal cut-off 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.
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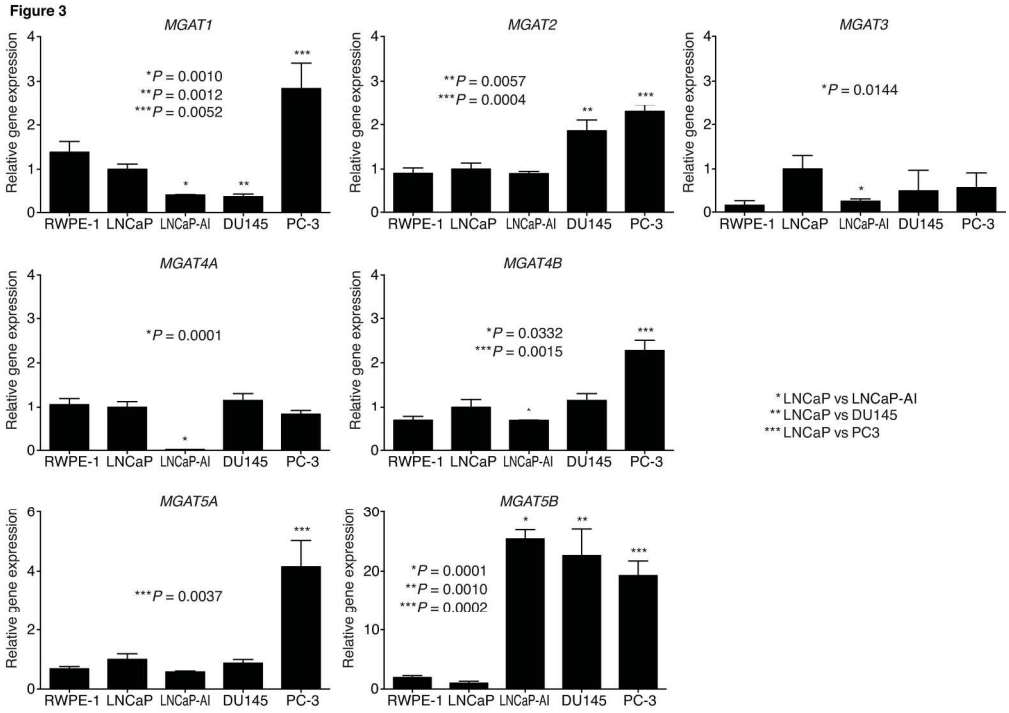

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205x145mm (300 x 300 DPI)

Table 1 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.

Table 2 Results of serum *N*-glycomics were significantly different between PC with ADT and 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)

Table 3 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

Table 4 Optimal cut-off levels of *m/z* 3049 and *m/z* 3414, sensitivity, specificity, accuracy, and predictive value.

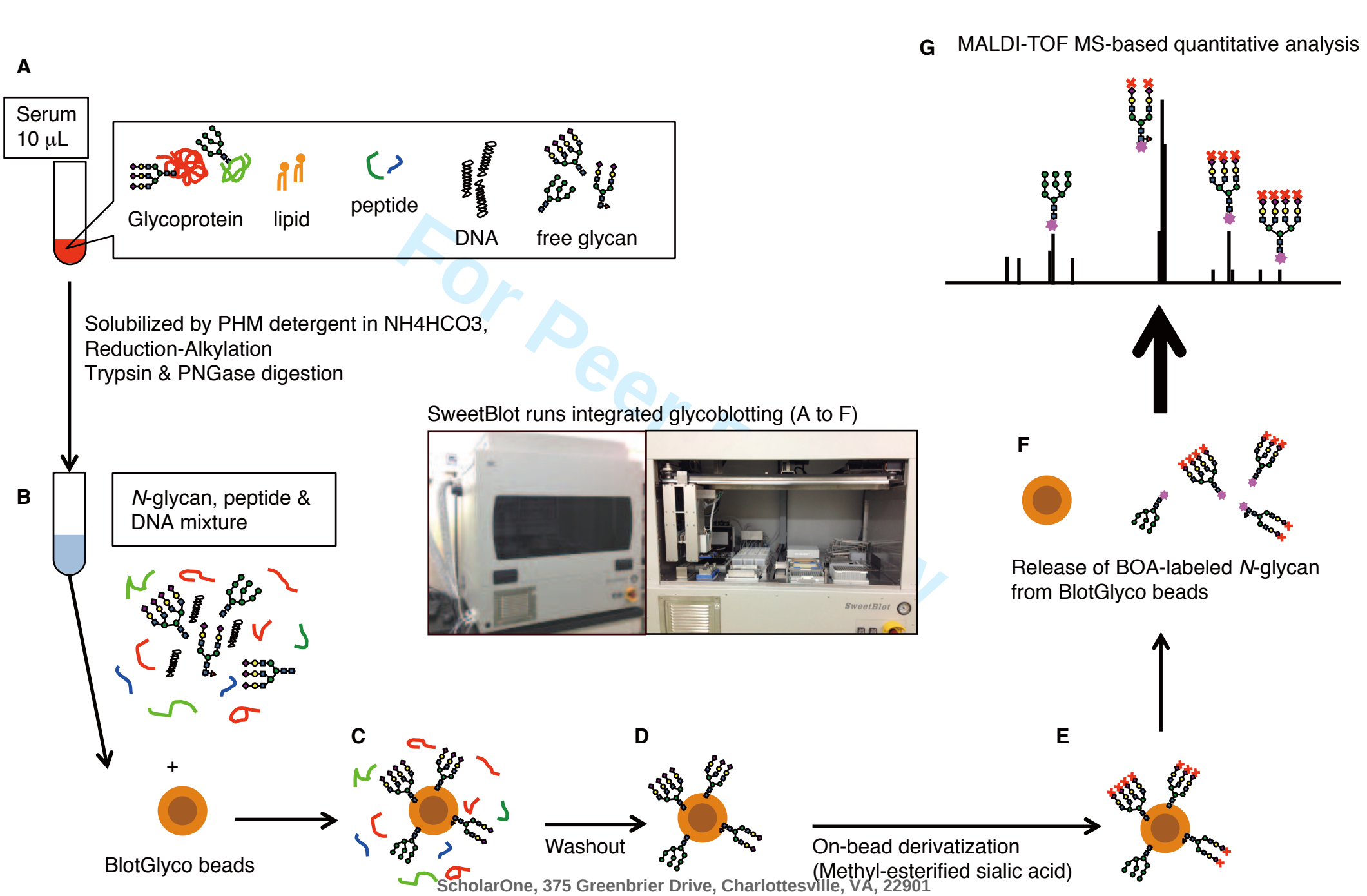
m/z	Cut-off (μM)	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)
3049	>1.595	69.1	37.0	56.1	61.8	44.7
3414	>1.355	69.1	41.3	57.9	63.5	47.5

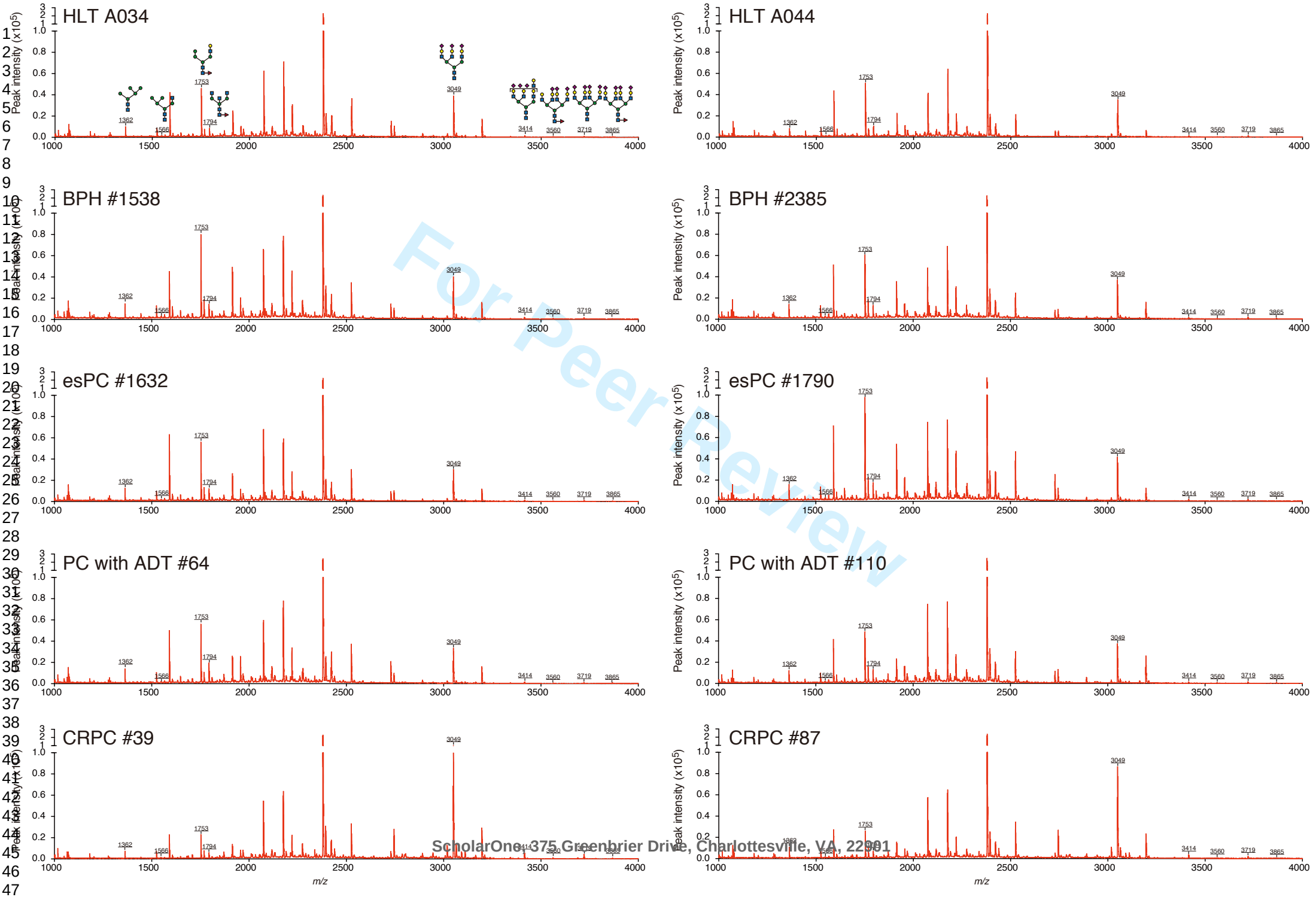
PPV: positive predictive value; NPV: negative predictive value.

Supplementary Table 1. Thirty-Six types of *N*-glycans demonstrated good quantitative reproducibility in all samples and could be analyzed statistically. *m/z* 2348.9 is the internal standard, disialo-galactosylated biantennary *N*-glycan, that contains amidated sialic acids (A2 amide glycans). Compositional annotations and putative structures are shown as abbreviations. Hex: hexose; HexNAc: *N*-acetylhexosamine; dHex: deoxyhexose.

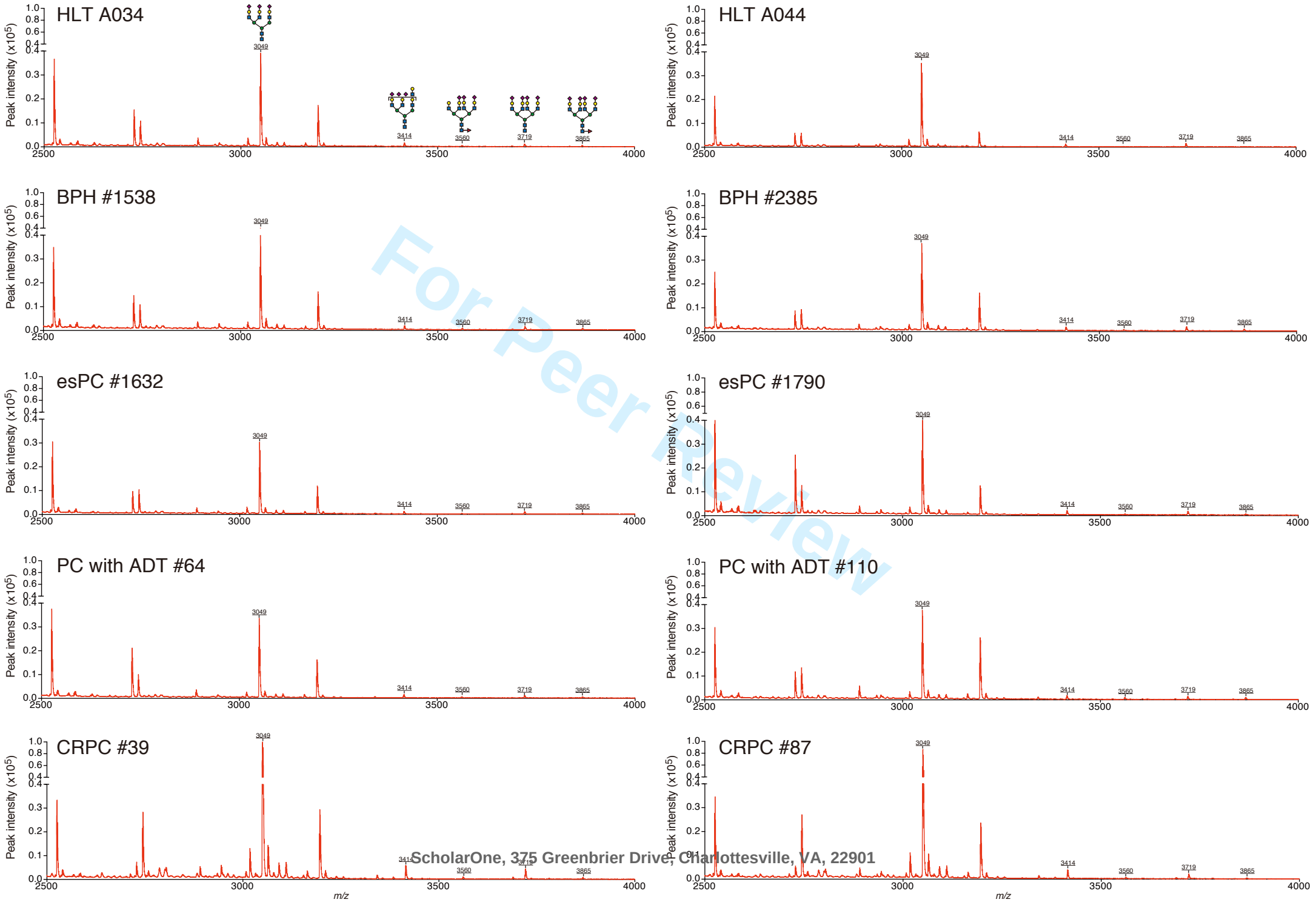
Peak No.	m/z	Composition
1	1362.5	(Hex)2 + (Man)3(GlcNAc)2
2	1524.5	(Hex)3 + (Man)3(GlcNAc)2
3	1565.5	(Hex)5 + (HexNAc)3
4	1590.6	(HexNAc)2(dHex)1 + (Man)3(GlcNAc)2
5	1606.6	(Hex)1(HexNAc)2 + (Man)3(GlcNAc)2
6	1647.6	(HexNAc)3 + (Man)3(GlcNAc)2
7	1686.6	(Hex)4 + (Man)3(GlcNAc)2
8	1708.6	(Hex)1(HexNAc)1(NeuAc)1 + (Man)3(GlcNAc)2
9	1752.6	(Hex)1(HexNAc)2(dHex)1 + (Man)3(GlcNAc)2
10	1768.6	(Hex)2(HexNAc)2 + (Man)3(GlcNAc)2
11	1793.7	(HexNAc)3(dHex)1 + (Man)3(GlcNAc)2
12	1809.7	(Hex)1(HexNAc)3 + (Man)3(GlcNAc)2
13	1848.6	(Hex)5 + (Man)3(GlcNAc)2
14	1870.7	(Hex)2(HexNAc)1(NeuAc)1 + (Man)3(GlcNAc)2
15	1914.7	(Hex)2(HexNAc)2(dHex)1 + (Man)3(GlcNAc)2
16	1955.7	(Hex)1(HexNAc)3(dHex)1 + (Man)3(GlcNAc)2
17	2010.7	(Hex)6 + (Man)3(GlcNAc)2
18	2032.7	(Hex)3(HexNac)1(NeuAc)1 + (Man)3(GlcNAc)2
19	2057.8	(Hex)1(HexNAc)2(dHex)1(NeuAc)1+ (Man)3(GlcNAc)2
20	2073.8	(Hex)2(HexNAc)2(NeuAc)1+ (Man)3(GlcNAc)2
21	2219.8	(Hex)2(HexNAc)2(dHex)1(NeuAc)1 + (Man)3(GlcNAc)2
22	2336.9	(Hex)3(HexNAc)4 + (Man)3(GlcNAc)2
23	2348.9	Internal standard (BOA-labeled A2 amide)
24	2378.9	(Hex)2(HexNAc)2(NeuAc)2 + (Man)3(GlcNAc)2
25	2524.9	(Hex)2(HexNAc)2(dHex)1(NeuAc)2 + (Man)3(GlcNAc)2
26	2727.9	(Hex)2(HexNAc)3(dHex)1(NeuAc)2 + (Man)3(GlcNAc)2
27	2743.9	(Hex)3(HexNAc)3(NeuAc)2 + (Man)3(GlcNAc)2
28	2890.1	(Hex)3(HexNAc)3(dHex)1(NeuAc)2 + (Man)3(GlcNAc)2
29	3049.1	(Hex)3(HexNAc)3(NeuAc)3 + (Man)3(GlcNAc)2
30	3109.1	(Hex)4(HexNAc)4(NeuAc)2 + (Man)3(GlcNAc)2
31	3195.2	(Hex)3(HexNAc)3(dHex)1(NeuAc)3 + (Man)3(GlcNAc)2
32	3341.2	(Hex)3 (HexNAc)3 (Deoxyhexose)2 (NeuAc)3 + (Man)3(GlcNAc)2
33	3414.2	(Hex)4(HexNAc)4(NeuAc)3 + (Man)3(GlcNAc)2
34	3560.3	(Hex)4(HexNAc)4(dHex)1(NeuAc)3 + (Man)3(GlcNAc)2
35	3719.3	(Hex)4(HexNAc)4(NeuAc)4 + (Man)3(GlcNAc)2
36	3865.4	(Hex)4(HexNAc)4(dHex)1(NeuAc)4 + (Man)3(GlcNAc)2

Supplementary Figure 1





Supplementary Figure 3



Supplementary Figure 1. General protocol for the integrated glycoblotting technique and workflow for glycoblotting-based high-throughput clinical glycan analysis. Ten-microliter serum samples (A) were applied to SweetBlot™ (System instruments, Hachijo, Japan) for glycoblotting. After enzymatic cleavage from serum protein, total serum *N*-glycans released into the digestion mixture (B) were directly mixed with BlotGlyco H beads (Sumitomo Bakelite, Co., Tokyo, Japan) to capture *N*-glycans (C). After the beads had been separated from other molecules by washing (D), sialic acid was methyl-esterified (E). These processed *N*-glycans were then labeled with benzyloxyamine (BOA) and released from BlotGlyco H beads (F). Mass spectra of BOA-labeled *N*-glycans were acquired using an Ultraflex III instrument (Bruker Daltonics, Germany) (G).

Supplementary Figure 2. Representative MALDI-TOF MS spectra (range of m/z 1000 to 4000) of BOA-labeled *N*-glycans derived from HLT, BPH, esPC, PC with ADT and CRPC patient serum. Significantly different *N*-glycans (*m/z* 1362, 1566, 1753, 1794, 3049, 3414, 3560, 3719, and 3865) between CRPC and other groups were shown in mass spectra. Symbols: yellow circles, galactose (Gal); green circles, mannose (Man); blue squares, *N*-acetylgulucosamine (GlcNAc); purple diamonds, *N*-acetylneuraminic acid (Neu5Ac); Red triangle, Fucose (Fuc).

Supplementary Figure 3. Representative MALDI-TOF MS spectra (range of m/z 2500 to 4000) of BOA-labeled *N*-glycans derived from HLT, BPH, esPC, PC with

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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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ABSTRACT

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.

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

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 become

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), early-stage PC (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 1.

Glycoblotting Method and Mass Spectrometry

Serum *N*-glycan analysis was performed as described previously using SweetBlot™ (System Instruments, Hachijo, Japan) [17] (Supplementary Figure 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 1), 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, USA) 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, 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, USA). 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, USA) by transiminization and were eluted in 150 μL of water. The BOA-labeled 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 0.5x, 0.75x, 1x, 1.25x, 1.5x, 1.75x, 2x, and 2.25x 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 abovementioned 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 ver. 20.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6.03 (GraphPad Software, San Diego, CA, USA). 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, USA) under the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. 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 (*G□□□□*) (Hs.PT.39a.22214847) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). 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 1) had good quantitative

reproducibility among all samples and could be used for statistical analysis. Table 1 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 2, 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 *m/z* 3049 and *m/z* 3414 were selected as specific *N*-glycans for the prediction of CRPC (Table 3). Fig.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 4). 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, B). Total PSA and testosterone levels were also followed-up at the same time points (Fig. 2C, D). We found that one PC 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. 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 Z 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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FIGURE LEGENDS

Figure 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).

Figure 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.

Figure 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 vs LNCaP-AI. Double asterisk symbol indicate *P* value of LNCaP vs DU145. Triple asterisk symbol indicate *P* value of LNCaP vs PC-3.