N- and O-glycome analysis of serum and urine from bladder cancer patients using a high-throughput glycoblotting method

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Abstract

Purpose: To develop novel diagnostic biomarkers for Bladder Cancer (BC), we concurrently evaluated serum and urine glycans in BC patients by utilizing a recently established glycoblotting method.

Experiments: N- and O-glycan levels in whole serum from 45 and 13 male patients diagnosed with BC were analyzed. As a control, 29 and 10 patients with benign prostatic hyperplasia (BPH) were also studied and the results were compared. Furthermore, urine N- and O-glycome from 8 patients with muscle-invasive BC and 11 with BPH were analyzed. In serum N-glycome analysis the area-under-the-curve (AUC) value was calculated by in house R software. In the other cases JMP, version 10.0.2 software package (SAS, Cary, NC) was used and p < 0.05 was considered statistically significant.

Results: The expression level of three N-glycans significantly increased in sera of BC patients. All of them had highly branched and sialylated structures with core-Fuc. The levels of three O-glycans were significantly higher in BC than in the control. In examination of urine samples, 16 N-glycans were significantly elevated in BC as compared to the control. Although 11 O-glycans were detected in urine samples, there was no significant difference in the expression levels.

Conclusions: The levels of highly branched, sialylated N-glycans and early sialylated O-glycans were increased in sera of BC patients. Moreover, we found that N-glycans increased in urine more than in serum of BC patients. These results suggest that the glycome change in urine directly results from glycoproteins that exist in BC cells. Thus, further large-scale glycan profiling will provide novel biomarkers for diagnosing BC in the near future.

Keywords: Bladder Cancer; Glycoblotting; N-Glycan; O-Glycan; Serum Biomarker; Urine Biomarker


Introduction

The human body consists of approximately 60 billion of cells, which are totally covered with carbohydrate chains. Generally glycans exist to combine with proteins and lipids. It is estimated that over 70% of all human proteins are glycosylated [1]. Glycans are known to have crucial roles in the molecular recognition and adhesion between cells and the disorder of this function affects greatly to the abnormality in human body. For instance, it has already been known that aberrant glycosylation of cell surface glycoproteins occurs in essentially almost all types of human cancers [2]. There are nine kinds of monosaccharides forming very complicated glycan structures, thus the information from glycans has great varieties compared with that of nucleotides and proteins. In addition, glycosylation is an event of post-translational modification that is not controlled directly by gene. Thus, glycans cannot be amplified in vitro by the polymerase chain reaction (PCR). Moreover, since there are various biomolecules such as nucleotides, proteins, lipids and intermediate metabolites in biological samples such as serum and tissue, it is extremely difficult to extract glycans with high-purity from small amount of those samples. In order to resolve this problem, a new technology for glycan-specific enrichment, the “glycoblotting method”, was developed recently [3-7]. Utilized by this method, high-throughput and quantitative glycomics can be performed on various biological samples that include large amount of impurities.

Oligosaccharides which bind onto proteins fall into two defined categories: N-glycans that linked to asparagine and O-glycans that linked to serine or threonine residue. While N-glycans are able to be released from protein backbone by peptide N-glycanase-F or –A [8,9], there is no enzyme acting to release O-glycans universally. Therefore, the main analyses of glycan structure have been focused on N-glycan. However, large-scale and high-throughput analysis of O-glycan is also required because an association of O-glycans in tumorigenesis has been of growing interest recently [10].

Bladder cancer (BC) is one of the most common cancers in humans and its incidence has been increasing during the past years. For diagnosis of BC, cystoscopy, urine cytology and computed tomography (CT) are commonly used in the clinic. However, cystoscopy is a painful maneuver with large aggression and the sensitivity of urine cytology...
for low-grade BC is ordinary low [11]. In addition, small tumor cannot be detected by CT. In cases with metastasis, the effectiveness of chemotherapy is just presumed by CT. In some cases, elevated level of serum Carcinoembryonic antigen (CEA) and Carbohydrate-antigen 19-9 (CA19-9) have been demonstrated in transitional cell carcinoma (TCC) of BC [12]. However, these markers are not routinely used in the clinic because of low specificity for BC. Similarly, several urine-based molecular tests including the measurement of soluble proteins such as bladder tumor associated antigen (BTA), nuclear matrix protein 22 (NMP22), proteins detected on fixed urothelial cells (ImmunoCyt) and chromosomal aberrations detected by fluorescence in situ hybridization (UroVysion) have been established. The advantage of urine analyses is the easiness of repeat sampling as well as the direct revelation of cancer tissue especially in BC. While sensitivities can be better for BTA and NMP22 over cytology, the specificity is impacted by other non-malignant conditions [13-16]. Although sensitivities of those assays are in the range of 75%-89%, the specificities are quite low. Because those tests are cellular assay depending on the amount of tumor cells, they are not suitable for the detection of low-grade and low-stage tumors.

For those reasons, exploration of useful markers for BC detection and monitoring of therapeutic effect is strongly required. Although several glycomics studies for BC have been reported [17,18], these are the results of human BC cell line or cancer specimen. In addition, they mainly focused only on the expression level of mRNA coding glycosyltransferases, but not on the changes in the amount and profiles of existing glycan. It is impossible to apply those results to therapeutics clinically unless the analyses are applied on samples such as blood and urine from BC patients.

In this study, we analyzed serum and urine N- and O-glycome of BC patients using glycoblotting method and evaluated the potential usage of glycans as novel clinical biomarkers for BC.

Materials and Methods

Patients

Serum N-glycome analysis was performed on 45 male patients diagnosed with BC at Department of Urology, Sapporo Medical University School of Medicine (Sapporo, Japan) between January 2011 and December 2012 (Table 1A). The mean ± standard deviation age of BC group was 70.4±8.99(range 51-93). 13 patient diagnosed with NMIBC, and December 2012 (Table 1A). The mean ± standard deviation age of BC group was 70.4±8.99(range 51-93). 13 patient diagnosed with high-grade NMIBC who were failed for conservative treatment (Table 1B). In addition to 10 patients with MIBC, 3 patients with nonmuscle-invasive BC (NMIBC) who were failed for conservative treatment or with micropapillary valiant in resected specimen underwent radical cystectomy. Histopathological examination revealed TCC in all, five of pT2, five of pT3 and two of pT4 tumors, including two of pT3 and one of pT4 patients had pathological lymph node metastasis. Two of pT2a patients were not indicated to radical cystectomy due to the advanced age and some complications and eventually underwent extra beam radiation therapy after transurethral resection (TUR). In addition, one patient with pT1(clinically T2) did not hope any treatments and only TUR was undergone for the purpose of hematuria control. For patients diagnosed high-grade NMIBC, intravesical BCG therapy following 2nd TUR were selected. Tumor staging was based on the TNM classification and the tumor grade was classified according to the WHO system. All serum samples of this patient group were collected before treatment.

Urine from 8 male patients with MIBC at Department of Urology, Sapporo Medical University School of Medicine (Sapporo, Japan) between January 2011 and December 2012, were also applied to N- and O-glycome analysis (Table 1C).

As a control for serum N-glycome analyses, 29 male patients with benign prostate hyperplasia (BPH) were selected (Table 1A). The mean...
± standard deviation age of the control group was 66.1±5.87 (range 55-79) and there was significant difference in each group (p=0.026). From these 29 patients, age-matched 10 and 11 patients were selected as the control of serum O- urine N- and O- glycomics(Table 1B, 1C). This study protocol confirmed to the ethical guidelines of the World Medical Association Declaration of Helsinki and was approved by the institutional review board. Informed consent was obtained from all sample donors.

Pre-treatment for Serum N-glycomics

Whole serum sample was treated according to the previous reports [4,5]. Serum N-glycome was analyzed by SweetBlotTM (System Instruments, Hachioji, Japan), an automated machine for pretreatment and glycoblotting. Briefly, 10 µl of serum samples containing 40 pmol of the internal standard, disialo-galactosylatedbiantennary N-glycan which carries amidatedsialic acids (A2 amide glycans), were allowed to reduction-alkylation by DTT and iodoacetamide (Wako Pure Chemical Industries, Osaka, Japan). Then the resulted mixture was trypsinized followed by heat-inactivation. After cooling down to room temperature, Peptide N-glycanase F (New England BioLabs, Ipswich, MA) was added into the mixture, to release the total serum N-glycans. After incubating for overnight at 37°C, 20 µl of the resulted solution, equivalent to 2.5 µl of serum, which was directly used for glycoblotting utilized by BlotGlycoH beads (Sumitomo Bakelite, Co., Tokyo, Japan) as described below.

Pre-treatment for Serum O-glycomics

We have already established a general protocol for releasing O-glycans on the basis of ammonia salts, which was modified the conventional alkaline conditions and optimized for serum O-glycomics system [19]. Serum samples (20 µl) were added 25 mg of dry powder of ammonium carbamate in 1.5 ml tubes. The mixture was incubate at 60°C for 20 h followed by additional of 500 µl water and evaporated at 60°C. The residual materials were reconstituted in 500 µl of 150 mM aqueous acetic acid and evaporated at room temperature. To the residue were added water and an internal standard (50 pmole of chitotetorase, GlcNAcβ1,4GlcNAcβ1,4GlcNAcβ1,4GlcNAc) and finally it was compromised to 40 µl. An aliquot of the solution was subjected to the glycoblotting as described below.

Pre-treatment for Urine N-glycomics

100 µl of urine samples were treated as the same way to serum samples basically. After releasing total urine N-glycans by PNGase F, the resulted solutions were completely dried up by SpeedVac. After addition of 20 µl of water and 21 pmole of the internal standard disialo-galactosylatedbiantennary N-glycan which carries amidatedsialic acids (A2amide glycans) to dried samples, concentrated samples including urine N-glycans were applied to the glycoblotting.

Pre-treatment for Urine O-glycomics

80 µl of urine samples were allowed to be added 60 mg of dry powder of ammonium carbamate and incubated at 60°C for 20 h following by additional 60 mg of ammonium carbamate and incubated 60°C for 20 h. After cooling down on ice, 200 µl of water and 200 µl of acetic acid (AcOH) were added and evaporated at room temperature. 40 µl of water containing 100 µmole/l of GN4 were added to dried samples. An aliquot of the solution was subjected to the glycoblotting as described below.

Glycoblotting

The aliquot of each sample was applied to the reaction with the BlotGlyco H+ beads in the subscribed condition to capture the glycans via stable hydrazone bonds. Then acetyl-capping of unreacted hydrazide functional groups on the beads and methyl esterification of the sialic acid carboxyl groups, existing in the terminal of the captured glycans, were performed sequentially, followed by serial wash before each step, as described before [19-25].The captured N-glycans were labeled with benzyloxylamine (BOA, Tokyo Chemical Industry, Tokyo, Japan) by trans-iminization and eluted with 100 µl of water. The BOA-labeled glycans were detected by MALDI-TOF/MS (Ultraflex 3 TOF/TOF mass spectrometer, Bruker, Germany).

Statistical analysis

The N-glycan and O-glycan peaks detected in MALDI-TOF/MS were picked using FlexAnalysis version 3 software (BrukerDaltonics). The compositions and structures of glycans were suggested by GlycoMod Tool (http://br.expasy.org/tools/glcomod). In case serum N-glycome analysis the area-under-the-curve (AUC) value was calculated by in house R software. In the other cases JMP, version 10.0.2 software package (SAS, Cary, NC) was used for the analysis. The Wilcoxon test was used to compare the expression level in each groups and p <0.05 was considered significant because they do not have enough number of samples to be assessed by AUC.

Reproducibility test of Sweetblot

We checked reproducibility of the N-glycomics performed by SweetBlot as described below. The serum samples, including BC, control and a serial dilution line of standard human serum (Sigma-Aldrich, St. Louis, MO) were set onto the plate in the machine and applied to the whole of the running program, i.e., pre-treatment, glycoblotting and MALDI-TOF/MS. After that all the same samples were analyzed again from the first step but with distinct position on the plate. The obtained former and later results were plotted as X-axis and Y-axis, respectively, for each glycan peaks detected in MALDI-TOF/MS. The obtained slope, correlation coefficient (R) and the number of the samples which showed appearance of corresponding glycans were shown in Table 1. Glycans with R>0.8 were thought to have a reproducibility of the detection in the serial experiments. After checking the reproducibility of such qualitative aspect, we also confirmed the quantitative reliability in each SweetBlot experiment. Peak area of each glycan detected in 0.5x, 0.75x, 1x, 1.25x, 1.5x, 1.75x, 2x and 2.25x concentration were plotted and judged quantitative reliability based on our own parameter. This assay was repeated twice as the same time to the qualitative reproducibility test. Only when satisfied our criteria in the both assay the glycan peak were judged as useful. Then we carefully looked for and removed the possibility of sub product from glycoblotting reaction, which should show the same m/z as detected glycans. The resulted glycans were used for statistical analysis.

Results

Reproducibility test of Sweetblot

Serum N-glycome analysis utilized by SweetBlot revealed that 50 kinds of BOA-labeled N-glycans were identified in the sera from both of the groups. In order to assess which glycans were able to be applied for statistical analysis, we performed the reproducibility test both from quality and quantitative aspect, of the glycoblotting utilized by SweetBlot as described in above. We found that 38 peaks corresponding to glycans, which is represent in Bold, showed good reproducibility (R>0.8) and allowed to use them for statistical analysis.
Serum N-glycome

Among all the N-glycans which are confirmed as reproducibility in detection the expression level of three glycoforms was elevated (m/z 2890: AUC=0.705, m/z 3560: AUC=0.714, m/z 3865: AUC=0.711, Figure 1) in BC. All of them had core-fucosylated and more than disialylated structures and two of three (m/z 3560 and 3865) were tetra-antenna glycans.

Serum O-glycome

Three major O-glycans, m/z 816, m/z 978, m/z 1121, which were presumed as (Gal)(GalNAc)(NeuAc), (Gal)2(GalNAc)(NeuAc) and (Gal)3(GalNAc)(NeuAc)2, respectively, were detected in all serum samples. The levels of these O-glycans was significantly higher in BC compared than in the control (m/z 816: p=0.030, m/z 978: p=0.001 and m/z 1121: p=0.002, Table 2). One typical O-glycan structure detected at m/z 1121 was significantly increased in BC throughout biosynthetic pathway (p=0.011, Table 2).

Urine N-glycome

N-glycome analysis using Glycoblotting method revealed that 32 of BOA-labeled N-glycans were expressed in urine samples from both of the groups (Table 3). Total amount of detected N-glycans was significantly increased in BC (p=0.003). Among those 32 glycans the expression levels of 16 glycans were significantly elevated in BC. Moreover, 8 of 16 increased glycans (m/z 1793, 1955, 2073, 2422, 2727, 3049 and 3719) in BC were verified the elevation in each biosynthetic pathway. In addition, among these 8 increased glycans 5 had core-fucosylated structures and 4 of 5 had bisected structure. The glycotyping analysis based on the structural classification [25], revealed that total amount of bisected, tri-antennary, tri-
Urine O-glycome

Significantly elevated in BC (p=0.007, 0.004, 0.023, 0.009, 0.006, 0.007, di-sialylated, tri-sialylated and tetra-sialylated structures were antennary with bisected, tetra-antennary, core-Fuc, mono-sialylated, and 32 N-glycans (m/z 656, 774, 816, 857, 876, 1019, 1121, 1168, 1181, 1546 and 1911, Table 4) were detected in all urine samples. However, the expression levels of the each 11 glycans were not significantly difference in two groups. Although one glycan (m/z 1168) was significantly decreased in BC through a biosynthetic pathway (p=0.009), there was no significant decrease in the expression level (p=0.302).

Table 2: Results of serum O-glycan analysis.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Mean expression (p mole/l)</th>
<th>p-value*</th>
<th>Biosynthetic pathway †</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z 816</td>
<td>135.56</td>
<td>108.26</td>
<td>0.030</td>
</tr>
<tr>
<td>m/z 978</td>
<td>8.08</td>
<td>5.66</td>
<td>0.001</td>
</tr>
<tr>
<td>m/z 1121</td>
<td>29.08</td>
<td>19.62</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 4: Results of urine O-glycan analysis.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Mean expression (p mole/l)</th>
<th>p-value*</th>
<th>Biosynthetic pathway †</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z 656</td>
<td>61.49</td>
<td>42.40</td>
<td>0.343</td>
</tr>
<tr>
<td>m/z 774</td>
<td>576.73</td>
<td>364.30</td>
<td>0.127</td>
</tr>
<tr>
<td>m/z 816</td>
<td>1236.48</td>
<td>967.76</td>
<td>0.092</td>
</tr>
<tr>
<td>m/z 857</td>
<td>16.90</td>
<td>16.95</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td>m/z 876</td>
<td>100.02</td>
<td>83.06</td>
<td>0.967</td>
</tr>
<tr>
<td>m/z 1019</td>
<td>15.35</td>
<td>13.23</td>
<td>0.050</td>
</tr>
<tr>
<td>m/z 1121</td>
<td>74.73</td>
<td>55.30</td>
<td>0.302</td>
</tr>
<tr>
<td>m/z 1168</td>
<td>5.61</td>
<td>8.34</td>
<td>0.302</td>
</tr>
<tr>
<td>m/z 1181</td>
<td>12.60</td>
<td>11.93</td>
<td>0.967</td>
</tr>
<tr>
<td>m/z 1546</td>
<td>4.27</td>
<td>3.36</td>
<td>0.773</td>
</tr>
<tr>
<td>m/z 1911</td>
<td>2.15</td>
<td>1.13</td>
<td>&gt;0.999</td>
</tr>
</tbody>
</table>

Discussion

Recent our studies of clinical glycomics have indicated that glycoblotting method [4] makes possible rapid, large-scale glycome analysis of human sera with high reliability that can be employed to search for novel carbohydrate-related biomarkers in various diseases [3]. In 2008, we reported the feasibility of high-throughput and quantitative glycomics on the basis of this approach by investigating human serum N-glycan profiling of 83 hepatocellular carcinoma (HCC) patients and 20 healthy controls [7]. By employing the ratio of every two peaks among structurally identified that indicate significant difference between disease and control, the algorithm finally selected three combinations of N-glycan ratio features that distinguished, with 100% accuracy, the HCC from controls. Moreover, Kamiyama et al. identified novel N-glycan structures suited as prognostic markers of HCC by investigating 369 patients underwent primary curative hepatectomy and followed up for a median of 60.7 months [22]. Importantly, Miyahara et al. revealed that the expression level of N-glycans identified as potential prognostic markers in the above study [23] might be a novel diagnostic and prognostic factor in 85 consecutive HCC patients [23]. Nousoet al. also demonstrated the versatility of this method in the serum N-glycan profiling of 92 pancreatic cancer patients and 243 healthy volunteers [24]. It was uncovered that the expression level of 15 N-glycans was increased in the serum of pancreatic cancer patients and 10 of 15 were significantly up-regulated in cases with distant metastasis. Hatakeyama et al. recently reported the glycan marker for renal cell carcinoma, which can be applicable for diagnosis and prognosis, utilized by glycoblotting. This is the first report in the urological field [25]. Despite emerging importance of the discovery research of new class serum glycan biomarkers, at present, clinical glycomics study based on the “glycoblotting method” has been performed mostly in a few limited diseases in these alimentary system [23]. The present study, therefore, is the first comprehensive glycomics by using a standardized glycoblotting protocol to assess the potentials of serum N- and O-glycomes as novel diagnostic markers of BC.

It is clear that malignant transformation may often be accompanied by changes in both N- and O-glycosylation of serum abundant glycoproteins. Given that most known serum biomarkers such as CA19-3 (MUC1), α-fetoprotein (AFP), and prostate specific antigen (PSA) can be detected only by using the individual antibody due to the extremely low serum concentration when compared with abundant...
serum glycoproteins, alteration of the glycoforms targeting total serum glycoproteins is promising alternate and sensitive biomarkers because these glycans should be attached posttranslational manner through the general biosynthetic processes within the cells.

It has been already known that β1,6GlcNAc branching of N-glycans is often increased [26] and the chain length of mucin-type O-glycans is generally shorter in cancer patients, notably “aberrant O-glycans” [27]. Branching N-glycan structures, such as bisecting GlcNAc and third branching β1,6GlcNAc are produced by enzymes GlcNAc-transferase-III (GnT-III) and GlcNAc-transferase-IV or -V (GnT-IV or -V), respectively. In the N-glycan biosynthetic pathway, GnT-III and GnT-IV competes for the same acceptor substrate. Once becoming bisected, the action of GnT-IV and -V is inhibited and, thus, sialylated-T antigen-like structures are formed to inhibit the activity of GnT-III. Therefore, increased levels of tri- and tetra-antenna structures should correlate with a decrease in the abundances of bisected glycans [28,29]. Tetra-antenna glycans result from increased activity of the GnT-V, which has long been associated with cancer development and metastatic potential [25,30,31]. There are some reports that increased branching has been associated with alterations in the hexosamine cycle and activation of the AKT pathway [26,30-34]. Indeed, increased levels of tetra-antenna oligosaccharides were detected on hemopexin purified from serum samples provided by patients diagnosed with HCC [22,35]. The increased GnT-V expression has been thought to be closely related to distant metastasis in colon and brain cancer [36,37]. Some reports showed the increased core-fucosylation of α-fetoprotein in HCC [38,39] and increased fucosylation of haptoglobin in ovarian, lung, breast and pancreatic cancers [40,41]. The levels of N-glycans with core-fucose (Fuc) and α,2,3-linked sialic acid residues were significantly increased in prostate cancer [42]. Elevation of sialic acid concentration in serum has been observed in various malignancies. Marth et al. have also reported the difference in sialic acid levels benign and malignant tumors [43]. Azab et al. observed that α2,3-linked sialic acid residues were over-structured glycoprotein in colon and brain cancer [44]. Moreover, high level of α2,3-linked sialic acid residues appeared to be associated with metastatic potential of gastric cancer cells [45].

In the current study, it was demonstrated that the elevated serum N-glycans in BC had tri- or tetra-antenna chains with a core-Fuc residue. Interestingly, these N-glycoforms were constructed in a single biosynthetic pathway. Therefore, our results of serum N-glycome in BC were almost in agreement with previously reported data in cases for other malignancies [22,35,38-43,45].

The addition of N-acetyl-D-galactosamine (GalNAc) to serine or threonine residue is the first common step of the biosynthesis of O-glycans, resulting in the formation of Tn antigen. Subsequent addition of sialic acid by a α2, 6-sialyltransferase forms sialyl-Tn antigen. On the other hand, T antigen, also known as core 1 structure, is an O-glycan formed by the dissacharide, Gal-β1,3GlcNAc [24]. Additionally, core-fucosylation of α-fetoprotein in HCC and increased core-fucosylation of α-fetoprotein in HCC [38,39] and increased sialylation by two sialyltransferases toward core 1 (T antigen) as an acceptor substrate afford both sialyl-T and disialyl-T structures while core 2 structure is a competitive product from the same T antigen was not observed in case for BC patients. Our present results were well consistent with the results previously obtained in other malignancies by using conventional analytical method [10,46-50].

Here we have also performed N- and O-glycome analysis of whole urine for the first time. We found that urine N-glycome showed that most of them were the same to which were detected in serum. However glycan which revealing significant difference in their expression level in BC were distinct from which in sera. The glycome change in urine might be directly resulted from glycoproteins exist in malignant cells in bladder, which can be released from cells, while the other reflected by metabolic changes in serum, caused by tumor immune reaction. We have already shown that O-glycome of MUC1 in urine from two healthy donors exhibited quite similar O-glycan expression pattern with sialylated O-glycans (94% of total) with 7:3 ratio of core 1 to core 2 [15]. In this study we have detected sialylated O-glycans which can be presumed as core 1 or core 2 structures, as well as many other structures. This is because MUC1 is a minor glycoprotein, although small amount but much other kind of glycoproteins can be existing in urine. Here we may observe the total O-glycome which is released not only from MUC1 but all those glycoproteins.

Although we revealed several changes both of N-O-glycans expression in BC, there are still several limitations in this study. The present study was performed preliminarily using small numbers of patients. Moreover, we selected BPH patients without BC as control because it was designed for a hospital-based study. Thus, compare to healthy volunteers, there might be a bias. In addition, this study needs to confirm whether detected glycoform changes are directly reflected from those in BC cells/tissue or the results influenced by the alteration of homeostatic immune balance affecting glycoprotein metabolism during tumor proliferation. However, the result obtained here, i.e., glycome changes in sera of BC patients, is fascinating enough because only the changes of glycan expression level analyzed by cell lines or cultured tissues cannot conduct to the serum tumor marker forever. From this view point, this study is more close to clinical application compared to previous studies for exploring BC tumor makers. Moreover, the preliminary data obtained by the present pilot study involved in a small sample number of BC patients should be assessed carefully by following large-scale glycomics studies.

In conclusion, the first serum N- and O-glycome analysis from the same BC individuals was performed by glycoplotting method. It was correlated with the histological stage and peritoneal dissemination [50]. Regarding with BC, there is quite little report about the expression of T-antigen and sialyl-T antigen. Videira et al. reported that the expression of ST3Gal-I, one of 3 types of sialyltransferase that produces sialylated T antigen, increased in BC tissue [17]. This result suggested that tumors may have slightly increased expression of T antigens compared with normal urothelium while they failed to find a significant difference. Moreover, there was no data about the occurrence of T- and sialyl-T antigen in serum of BC patients.
demonstrated that the serum expression level of three N-glycans was up-regulated in BC. Moreover, the expression level of highly branched and/or heavily sialylated N-glycans were increased significantly in BC, as well as early sialylated O-glycans. These results were not contradicted to the previous reports in other cancers [10,18,30,33-38,40,42-45].

Based on the current study, further large-scale glycan profiling will provide noble biomarkers for diagnosing BC in the near future.

Acknowledgment
Dr. Yoshikazu Sato provided the advice of samples collection. Mrs. Shiori Ito provided the management of samples. This work was partly supported from a grant for “Discovery of new biomarkers and development of diagnostic method based on large-scale glycomics” by JSPS KAKENHI Grant Number 25220206 and “Development of Systems and Technology for Advanced Measurement and Analysis” by JST-SENTAN.

Conflict of interest statement
None of the authors have any conflicts of interest associated with this study.

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