Chapter 14

A Glycomics Approach to the Discovery of Potential Cancer Biomarkers

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Abstract

Glycosylation is highly sensitive to the biochemical environment and plays a key role in development and disease manifestation. Moreover, glycan biosynthesis depends on several highly competitive processes; thus, variations in the concentration of specific glycosyltransferases produce different products. For this reason, monitoring changes in glycosylation may be a more specific and sensitive approach to biomarker discovery and possibly disease diagnosis. Glycans in serum are of particular interest as approximately half of all proteins are glycosylated. We have developed the methods for profiling the glycans in human serum to identify glycan biomarker. Global release methods were used including chemical and enzymatic to access O-linked and N-linked glycans, respectively. Glycans were released from the culture medium of various cancer cell lines, in control sera, and in cancer patients and isolated using solid phase extraction (SPE) with a porous graphitized carbon. The SPE fractions were analyzed by matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI FTICR MS). Glycan compositions were determined based on accurate masses and tandem mass spectrometry. Glycosylation changes between control and patient group were monitored. Several glycans were identified as potential markers for ovarian, breast, and prostate cancer. In short, direct glycan analysis of human serum without any protein identification represents a new and innovative approach to disease marker discovery.

Key words: Glycan, glycosylation, MALDI, mass spectrometry, biomarker, serum.

1. Introduction

Changes in glycosylation have been implicated in many diseases including cancer (1–3). Glycosylation, whether O-linked or N-linked, is highly sensitive to the biochemical environment and is one of the most common post-translational modification of proteins particularly on the cell surface and in the extracellular

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matrix (4, 5). The biosynthesis of glycan relies on a number of highly competitive processes involving glycosyltransferases, suggesting that the expression of the glycans products is highly variable. Thus, monitoring changes in glycosylation may prove to be an innovative technique for biomarker discovery and eventually disease diagnosis by offering more specificity and sensitivity.

Lectin-based glycan detection methods (6–10) such as lectin affinity, immuno-affinity electrophoresis, and lectin blotting methods have been used for studying the roles of glycans in diseases. However, these approaches select for specific glycans or classes of glycans such as those that are sialylated or fucosylated. More recently, global glycome approaches have been explored as a source for disease markers. These approaches have been hindered by the complexities of glycan structures and the limitations in analytical methods for elucidating structures. With the advent of mass spectrometry, which provides a rapid quantitative tool for component analysis, the limitations are diminishing swiftly.

Global glycan profiling of human serum with matrix-assisted laser desorption/ionization (MALDI) (11–17) and electrospray ionization (ESI) (18–20) mass spectrometry has recently been explored and has already led to several potentially promising markers for several diseases. These approaches rely on the notion that many diseases cause aberrant glycosylation. Monitoring and diagnosing a specific disease will rely primarily on the changes in glycosylation with little regard for the specific protein to which the glycans may be bound (21). The additional advantage of this approach is that patient sera can be examined without any protein purification, while glycans from glycosylated proteins originating from the tumor or from circulating cancer cells are both simultaneously observed.

Initial glycan profiling efforts focused on the O-linked glycans, which are glycans attached to the serine (Ser) or threonine (Thr) residue of the polypeptide backbone. In this approach glycans were globally released from glycoproteins by reductive β-elimination (11, 12) and analyzed by matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance (MALDI FTICR) mass spectrometry. Mass profiles were used to examine changes between diseased and control patients. For ovarian cancer, 15 possible O-linked glycan markers were found in patient sera that were not found in normal sera (11, 22, 23). Several breast cancer tumor cell lines and patient sera were also analyzed for possible breast cancer "glycan signatures" (12). In these studies, many glycan masses were found to be derived from N-linked glycans, produced by the peeling reaction during the sodium hydroxide/sodium borohydrate treatment. β-Elimination therefore released both N-linked and O-linked glycans simultaneously. It was also determined that because of the relative abundances of the N-linked glycans, they contaminated

the O-linked glycan analysis. Nonetheless, there were distinct glycans that could be readily identified in the clinical setting that corresponded to the onset of the disease (22).

To complement the O-linked glycan release, the N-linked glycans were examined by selective release in ovarian, breast, and prostate cancer patient sera (14). N-Glycans have several advantages over O-linked glycans that make them an easier group to explore for disease markers (24). N-Linked glycans are significantly more abundant in human sera than the O-linked glycans. Their syntheses follow a set of well-prescribed rules, which make them perhaps an easier group to explore for disease marker. N-Linked glycans are released by enzymes under physiological conditions.

Gycans released by the enzyme PNGase F were profiled by MALDI FTICR MS providing high-mass accuracy and high resolution. The exact glycan compositions (hexoses, *N*-acetylhexosamines, sialic acids, and fucoses) were easily deduced based on accurate masses achieved by FTICR MS and by high-performance time-of-flight (TOF). Tandem mass spectrometry in the form of infrared multiphoton dissociation (IRMPD) and collision-induced dissociation (CID) were used to confirm composition and to obtain structural information of the potential biomarkers.

This chapter will illustrate the glycan profiling methods using global release of O-linked and N-linked glycans in human serum for the discovery of cancer biomarkers. These methods utilize chemical and enzymatic processes to release glycans from cell line supernatant and cancer patient sera.

2. Materials

2.1. Ovarian Cancer Cell Growth and Supernatant Acquisition

1. Caov-3, OVCAR-3, ES-2, and SK-OV3 ovarian cancer tumor cell lines were obtained from the American Type Culture Collection (ATCC).
2. Caov-3 and OVCAR-3 cancer cells were cultured in RPMI1640 cell medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin/streptomycin, and 1% glutamine. ES-2 and SK-OV-3 cells were grown in McCoy's medium.
3. Conditioned medium (CM, supernatant) was removed from the cells during log (non-confluent) or death (confluent) cell growth, and frozen at –70°C. The CM was thawed, sterile filtered (0.2 μ filter), and concentrated using Vivacell 70 or Vivaspin 20 concentrators (VivaScience, Edgewood, NY).

2.2. Human Serum Samples

1. All human serum samples were acquired from the University of California-Davis Medical Center Clinical Laboratories using an Internal Review Board (IRB) approved protocol.
2. For ovarian cancer, 48 ovarian cancer patients and 24 healthy controls were examined. The ovarian cancer patients were at various stages of the disease ranging from post-operative, under treatment with chemotherapy, at recurrence, and under surveillance. CA125 testing was conducted by the UC Davis Medical Center Clinical Laboratory using the CA125 assay kit (AXSYM test for CA125, Abbott, Abbott Park, IL).
3. For prostate cancer, 20 serum samples from individuals with prostate cancer ($n = 10$, under active surveillance for prostate cancer, PSA = 5.4-27.0 ng/mL) and individuals with their prostate removed ($n = 10$, post-radical retropubic prostatectomy (post-RRP), PSA<0.1 ng/mL).PSA testing was conducted by the UC Davis Medical Center Clinical Laboratory using the ADVIA Centaur PSA assay test on a Bayer ADVIA Centaur (Bayer Diagnostics, Tarrytown, NY).

2.3. Release and Purification of Oligosaccharides

1. Release solution for O-linked glycan: 1.0 M $NaBH_4$ in 0.1 M NaOH, prepared freshly.
2. Digestion buffer for releasing N-linked glycans: 200 mM ammonium bicarbonate (NH_4HCO_3) and 10 mM dithiothreitol (DTT).
3. Dialysis (Slide-A-Lyzer 10 kDa MWCO Dialysis Cassettes, Thermo Fisher Scientific Inc., Rockford, IL).
4. Neutralizing solution for β-reductive elimination: 1.0 M HCl.
5. Solid phase extraction (SPE) graphitized carbon cartridges (150 mg, 4 mL) from Alltech Associates, Inc. (Deerfield, IL).
6. Activation solution: nanopure water and 80% acetonitrile (AcN) in 0.1% trifluoroacetic acid (TFA) (v/v).
7. Washing solution: nanopure water.
8. Elution solutions: 10 and 20% AcN in H_2O and 40% AcN with 0.05% TFA in H_2O.

2.4. MALDI FTICR Analyses of Oligosaccharides

1. Mass spectra were recorded on an external source HiResMALDI (IonSpec Corporation, Irvine, CA) equipped with a 7.0 Tesla magnet. The HiResMALDI is equipped with a pulsed Nd:YAG laser (355 nm).
2. Matrix for mass spectrometry analysis: 2,5-dihydroxybenzoic acid (DHB) (5 mg/100 μL in 50% AcN in H_2O).
3. Positive mode dopant: 0.1 M NaCl in 50:50 H_2O/AcN.

3. Methods

The procedure, which included the release and isolation of the glycans, is outlined in **Fig. 14.1**. These streamlined methods were developed to minimize sample handling and increase speed. The glycans were released, pre-concentrated, and fractionated into solvents of differing polarity. The glycan mixtures eluted from three SPE fractions were mass profiled using MALDI FTICR MS. In this way, extensive chromatography is avoided while possible suppression of glycan signals by other glycan components is minimized. Because high-performance mass spectrometry is used, the glycan composition can be determined solely based on the masses (*see* **Note 1**).

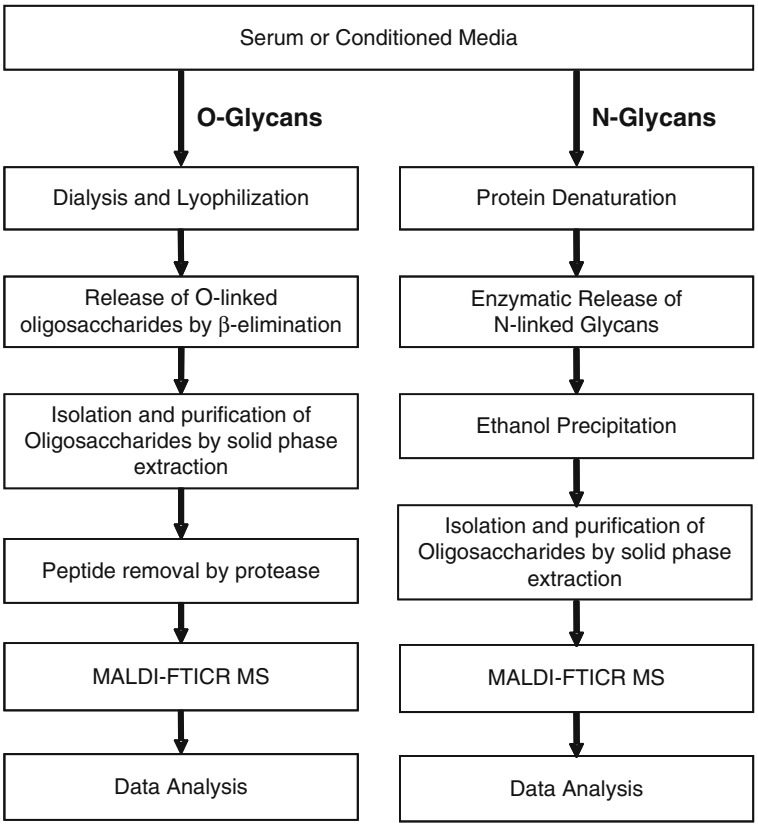

Fig. 14.1. The strategy for the release and isolation of glycans in the conditioned media of cell lines and human serum. Left pathway shows the release of O-linked glycan while right represents the N-linked glycan.

3.1. Release of O-Linked Glycans by β -Elimination

1. Serum samples (100 μL) were dialyzed against nanopure water at 4°C for 12–16 h and then lyophilized.

2. Alkaline borohydride solution (500 μL) was added to 2–3 mg of lyophilized serum samples.
3. The mixture was incubated at 42°C for 12 h in a water bath.
4. After the reaction, 1.0 M hydrochloric acid solution was slowly added to samples in an ice bath to stop the reaction and destroy the excess sodium borohydride.
5. Released glycans were desalted and fractionated prior to MS analysis.

3.2. Enzymatic Release of N-Linked Glycans

1. Human serum (100 μL) was added to 100 μL of digestion buffer.
2. The reaction mixture was heated to 100°C for 2 min to denature the protein.
3. After cooling at room temperature, 2.5 μL of Peptide *N*-glycosidase F (PNGase F) was added and the mixture (pH 7.5) was incubated at 37°C for 24 h.
4. Chilled 800 μL ethanol was added and the mixture was frozen for 1 h and then centrifuged. In the process proteins were collectively in a pellet and the glycans were in the supernatant.
5. The supernatant was completely dried down to remove the ethanol and purified by GCC-SPE.

3.3. Glycan Purification Using a Graphitized Carbon Cartridge-Solid Phase Extraction (GCC-SPE)

1. Prior to use, GCC was washed with deionized water and 80% AcN in 0.05% aqueous TFA (v/v).
2. Glycan solutions were applied to the GCC cartridge and subsequently washed with several cartridge volumes of deionized water at a flow rate of 1 mL/min for desalting.
3. Glycans were eluted with 10% AcN in H_2O (v/v), 20% AcN in H_2O (v/v), and 40% acetonitrile in 0.05% aqueous TFA (v/v).
4. Each fraction was collected and dried in a Centrivap apparatus. Fractions were reconstituted in nanopure water prior to MS analysis.

3.4. Mass Spectrometry Analysis

1. A saturated solution of NaCl in 50% AcN in H_2O was used as a cation dopant to increase signal sensitivity.
2. The glycan solution (1 μL) was applied to the MALDI probe followed by matrix solution (1 μL).
3. The sample was dried under vacuum prior to mass spectrometric analysis.

3.5. Data Analysis

3.5.1. Glycan Analysis of Ovarian Cancer Cell Lines

In this study, the conditioned medium of four ovarian cancer cell lines (Caov-3, OVCAR-3, ES-2, and SK-OV-3) in both log and death phases were extracted and examined. The glycans were released by β-elimination, i.e., in the presence of $NaBH_4$ and NaOH. This is the standard procedure for releasing O-linked glycans. Representative MALDI FTMS spectra of glycans found in the conditioned medium of ovarian cell lines in the positive mode are shown in **Fig. 14.2**. The four ovarian cancer cell lines had many glycans in common, but some (open circle) were unique to each cell line. Labeled peaks (solid circle) in the spectrum are prominent glycans present in all cell lines. Samples eluted with 40% AcN seem to provide the most abundant and largest glycans, using a positive mode on MS. They were identified to be neutral glycans based on accurate mass and tandem mass spectrometry.

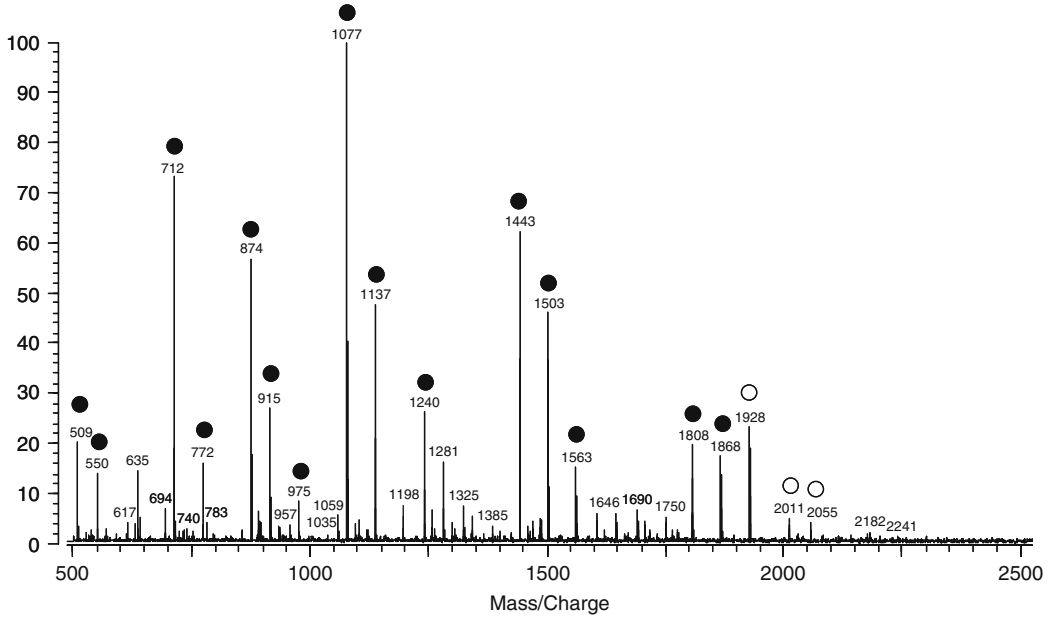

Fig. 14.2. Representative MALDI-FTMS spectra (positive mode) of glycans found in the conditioned media of ovarian cell lines released using alkaline sodium borohydrate. The majority of the peaks come from fragments of N-glycans due to peeling reaction. *Solid circle* represents common glycans in all cell lines while *open circle* represents cell-line specific glycans.

As controls, the complete medium containing 10% fetal bovine serum (FBS) as well as the defined medium without FBS was analyzed in a similar manner as the conditioned medium from the cell lines. From the complete medium containing 10% FBS, few glycans were observed, and from pure FBS only a small number of glycans were observed (less than five distinct masses). The abundances of these ions were significantly less than the cultured cell by as much as two orders of magnitude. The major ion peaks corresponded primarily to peptides whose masses were not observed in the cultured cell samples.

3.5.2. Glycan Profiling of Ovarian Cancer Patient Sera

In this small pilot study, human serum samples of five ovarian cancer patients and five healthy controls were examined to determine whether the same glycans observed in the conditioned medium of the ovarian cancer cell lines can be detected in ovarian cancer patient serum. Samples were treated with the same procedure as the cell lines. A typical mass spectrum of cancer patients and control group is shown in **Fig. 14.3**. The spectra from the patient sera contained many of the glycans found in the same fraction of the samples obtained from the ovarian cancer cell lines. In the normal sera, these signals are either totally absent or were present as only one or two peaks with very minor abundances. Tandem MS spectra were performed on the individual peaks and they were found to fragment and yield successive glycan losses.

To determine the feasibility of this approach on human serum, the glycan analysis was expanded to a larger set of serum samples consisting of 48 ovarian cancer patients and 24 healthy individuals. The ovarian cancer patient samples were chosen primarily based on their CA125 levels (23 samples for low CA125: <35 U/mL, 25 samples for high CA125: >150 U/mL). The ovarian cancer patients were high heterogeneous at various phases in their disease course. Forty-four of 48 patients had same glycans (at least 16 glycans) found in ovarian cancer cell lines and small set of serum samples while 23 of 24 control subjects had no detectable glycans.

For better comparison and quantification, the mass signal intensities of a selected group of glycans (labeled peaks in **Fig. 14.3a**) were summed for both sets of samples. In **Fig. 14.4 t**he samples on the left, with little or no intensities, are the normal samples while those on the right are samples from cancer patients. It is readily apparent that patient groups have high levels of "signature" glycans, while control groups have low or absent levels of the same glycans.

Receiver operating characteristic (ROC) curves were performed on a larger set of cancer patients (both with high and low CA125) using the glycan markers and compared to the CA125 serum levels. The ROC curve represents a trade-off between sensitivity and specificity. The greater the area under the curve indicates the better the test performance of the diagnosis. The area under the ROC curve (25) for the glycan markers is 0.95 (95% CI: 0.896, 1.0) indicating a "very good" to "excellent" test, while those for CA125 was 0.67 (95% CI: 0.57, 0.79) indicating a "fair" test for ovarian cancer (**Fig. 14.5**).

3.5.3. Glycan Profiling of N-Linked Glycans from Prostate Cancer Patient Sera

In this study a small set of serum samples consisting of 10 patients and 10 controls were initially analyzed. Globally released glycans were profiled by enzyme PNGase F without the need for protein identification, separation, and purification. **Figure 14.6**

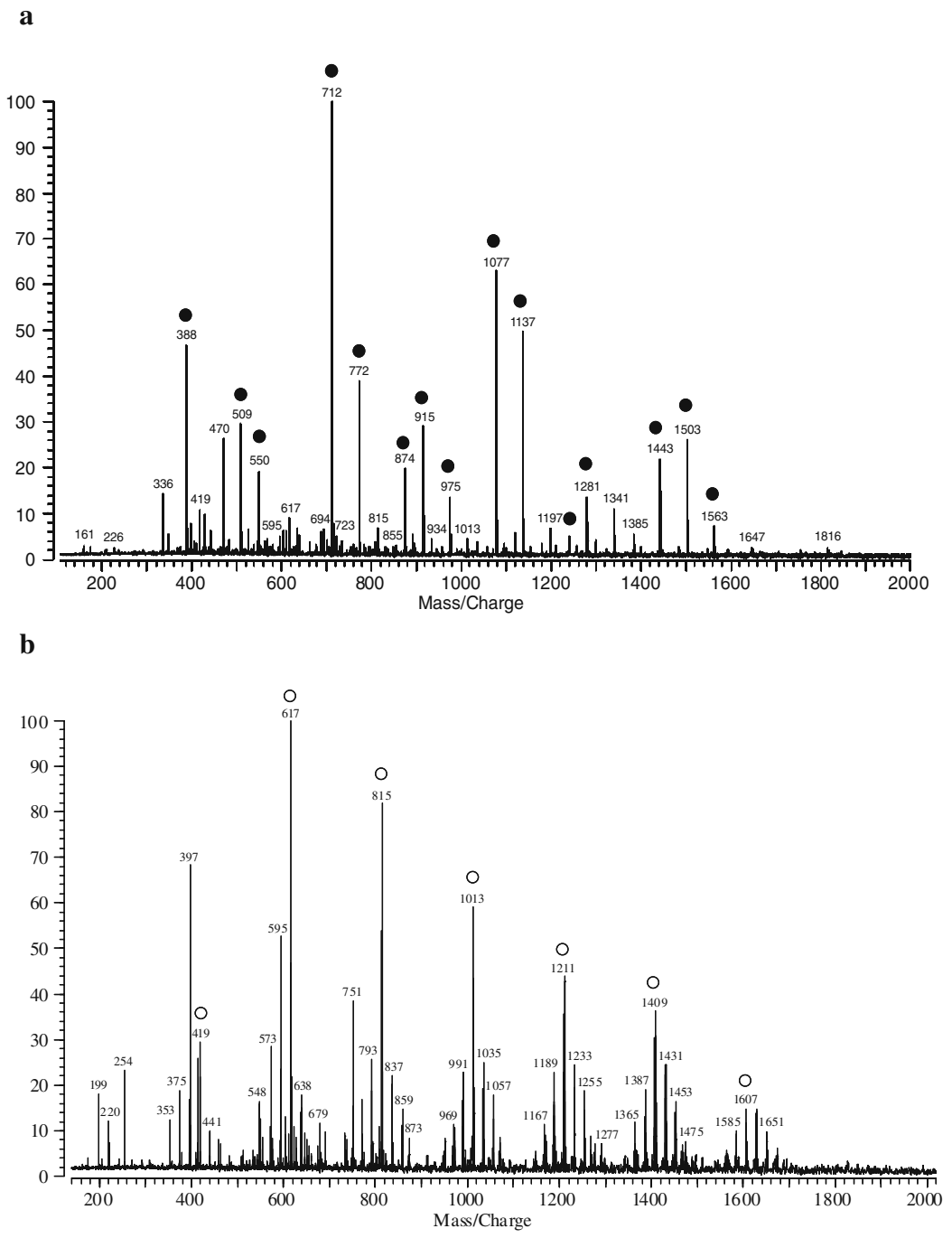

Fig. 14.3. Representative MALDI-FTMS spectra of (a) ovarian cancer patients and (b) normal individuals in the positive mode. *Solid circles* in (a) indicate glycans unique to ovarian cancer patients while *open circles* in (b) correspond to glycans found in both normal and cancer patients.

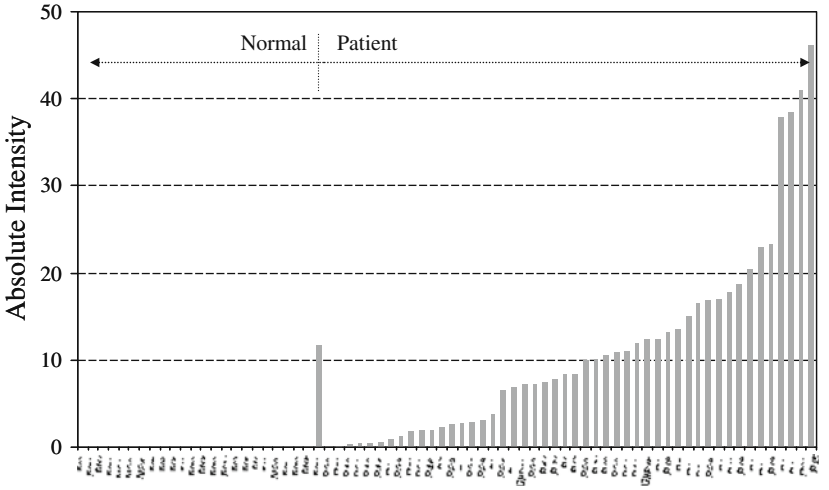

Fig. 14.4. Total intensities of glycans labeled in **Fig. 14.3a** for each individual (24 healthy controls and 48 patients with ovarian cancer). The levels of glycans were increased in ovarian cancer.

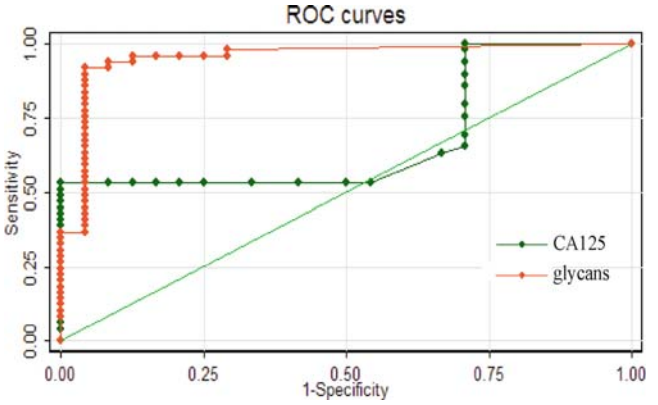

Fig. 14.5. Receiver operating characteristic (ROC) curves for all samples (48 ovarian cancer and 24 healthy control) using glycomics assay and CA125 value.

shows the representative spectra of the prostate cancer serum samples in 10, 20, and 40% fractions. N-Linked glycans are readily identified by trimannosyl core ($Man_3GlcNAc_2$) and accurate masses obtained from FTICR MS (*see* **Note 2**). The figure gives the structures of the abundant glycans only. Zooming in on the low-abundance regions resulted in exposure of more glycans. In the 10% AcN spectrum, the glycans are mostly the neutral ones in the low mass region and the scans are done in the positive mode. These are mostly the mannose and complex-type sugars. The acidic glycans in the higher mass region come out at 40% AcN and thus, the spectral scan is in the negative mode. The 20% AcN fraction contains the neutral and acidic glycans in the middle mass region and so the positive and negative scans were done on

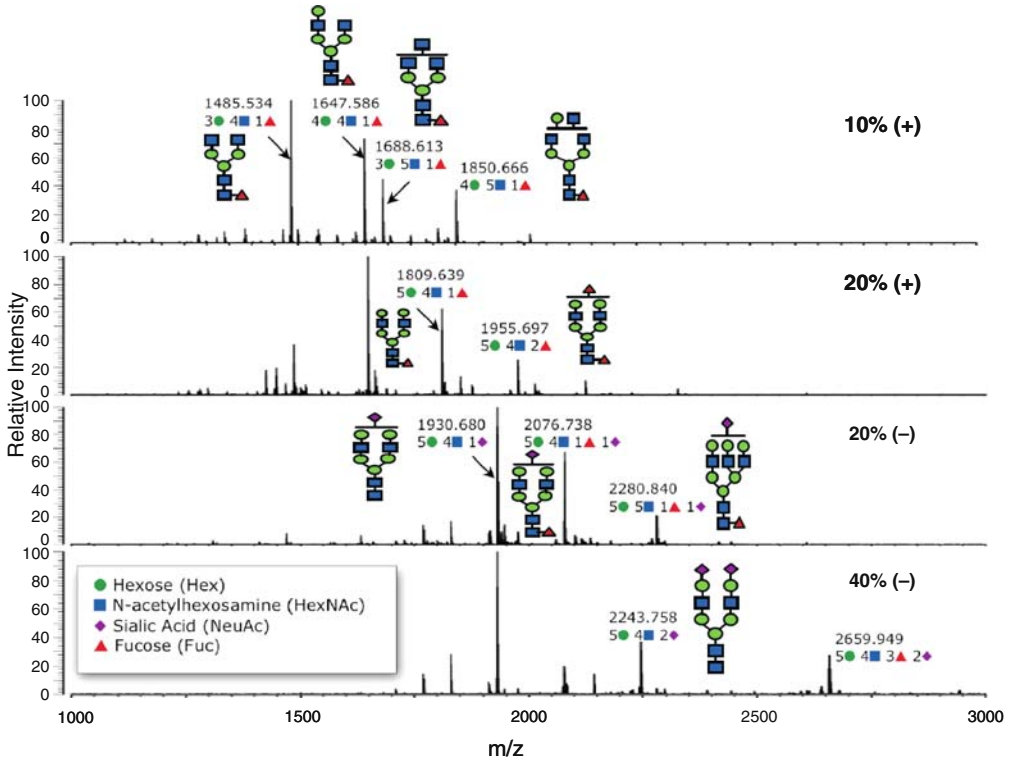

Fig. 14.6. Representative MALDI FT-ICR mass spectra of N-linked glycans in patient and control sera. 10, 20, and 40% represent glycans eluted with corresponding fraction of acetonitrile (AcN) in the positive (+) and negative (–) ion mode of the mass spectrometer. Structures are putative and are based only on accurate masses.

the said fraction. Thus, a progression from 10 to 40% AcN fractions shows not only an increase in glycan size but also in glycan polarity.

For the comparison of the peaks in patient and normal samples, statistical analysis using one-way analysis of variance (ANOVA) was performed. The absolute intensity of peaks was divided by the total ion intensity of the spectrum then multiplied by 1000. The normalized intensity of base peaks of each sample was in the range of 90–120. If the *p*-value was less than 0.05, the difference between the two groups was considered statistically significant. The calculations were carried out by the SYSTAT 11 (Systat Software Inc., San Jose, CA).

Seven potential N-linked glycan biomarkers (**Table 14.1**) were identified from the three SPE fractions. These were mostly high mannose type N-linked glycans. Four high-mannose (Man6–Man9) type, one neutral, and one acidic complex-type glycans are downregulated in the patient group while one acidic complex-type glycan is upregulated in the patient group with active disease (under active surveillance). These glycan changes are

Table 14.1
The seven possible N-linked glycan biomarkers that are statistically significant ($p < 0.05$). Glycan compositions are deduced from the accurate mass determined by MALDI-FTICR MS

m/z [M+Na]+	Composition	Type
1419.475	2HexNAc:6Hex	High mannose
1581.528	2HexNAc:7Hex	High mannose
1647.586	4HexNAc:4Hex:1Fuc	Complex/hybrid
1743.581	2HexNAc:8Hex	High mannose
1905.634	2HexNAc:9Hex	High mannose
1976.659	4HexNAc:5Hex:1NeuAc	Complex
2568.884	4HexNAc:7Hex:2Fuc:1NeuAc	Complex/hybrid

significantly different from individuals without active disease after having undergone RRP.

In many cases the overall profile of the abundant glycans especially for N-linked glycans is the same for the patient and control groups. A number of methods to determine the most reproducible and reliable glycan biomarkers in serum were developed in our laboratory. A glycan library was created to rapidly annotate composition in glycan mass profiles. Another method was used to analyze MALDI FTICR MS data. The MALDI FTICR MS data analysis process was performed in six steps: baseline correction, data transformation, peak location, peak identification, normalization, and statistical analysis (26). A shifted-log transformation was employed to stabilize the variance. Peak identification was performed on the shifted log scale using a quadratic curve fitting procedure. Although the m/z scale was used for instrument calibration, additional accuracy was obtained using a secondary calibration in which "strong" peaks – peaks that are large in all spectra – are made to correspond exactly in mass location. This procedure was done using a cubic interpolation spline (interpSpline in the splines package in the R software package) of the strong peaks. At this point, peaks in different spectra whose masses differed by at most 0.01 Da were considered as identical on the m/z scale. The spectra were additively normalized on the log scale using the average height of the strong peaks in the spectrum.

Statistical analysis to identify the glycan profiles for the cancer data was conducted using a one-way ANOVA model on each m/z set of peaks (corresponding to a compound). Since thousands of compounds were being generated for each glycan profile, multiple testing using the method of Benjamini and Hochberg

(27) was applied to control the false discovery rate (FDR) at 0.1. The Benjamini and Hochberg method can be highly sensitive to the set of starting *p*-values. Since biomarkers should be strongly present in at least one of the groups analyzed, those compounds that had a large peak in at least "K" spectra, where "K" was a number to be determined. For each data set, the set of significant differences was calculated for all possible values of K from one to the total number of spectra. As a check on the *p*-values obtained by these methods, a permutation test was performed on the peaks that were found to be significantly different to get a distribution-free estimate of the *p*-values. The results from the analysis for the prostate cancer samples show that the glycans were found to be statistically different between patients with prostate cancer and normal healthy controls.

4. Notes

1. N-Linked glycans are often released with an enzyme whereas O-linked glycans are released chemically. It is possible to release both types simultaneously but not with the desired completeness.
2. High mass accuracy and high resolution are advantages in profiling oligosaccharides. The capability of high-resolution mass analyzer such as Fourier transform ion cyclotron resonance (FTICR) to provide accurate mass has been crucial for obtaining basic glycan structure information. For example, an oligosaccharide with quasimolecular ion ($[M+Na]^+$) at m/z 2201.819 has three possible compositions within a tolerance of ± 0.1 mass units. Only with a tolerance of 0.01 mass units is the correct composition of two Fuc, four Hex, and six HexNAc obtained.

Acknowledgments

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References

1. Brockhausen, I. (1999) Pathways of O-glycan biosynthesis in cancer cells. *Biochim. Biophys. Acta* **1473**, 67–95.
2. Dube, D. H. and Bertozzi, C. R. (2005) Glycans in cancer and inflammation. Potential for therapeutics and diagnostics. *Nat. Rev. Drug Discov.* **4**, 477–488.
3. Fuster, M. M. and Esko, J. D. (2005) The sweet and sour of cancer: glycans as novel therapeutic targets. *Nat. Rev. Cancer* **5**, 526–542.
4. Apweiler, R., Hermjakob, H., and Sharon, N. (1999) On the frequency of protein glycosylation, as deduced from analysis of

the SWISS-PROT database. *Biochim. Biophys. Acta* **1473**, 4–8.
5. Dennis, J. W., Granovsky, M., and Warren, C. E. (1999) Protein glycosylation in development and disease. *Bioessays* **21**, 412–421.
6. Manning, J. C., Seyrek, K., Kaltner, H., Andre, S., Sinowatz, F., and Gabius, H. J. (2004) Glycomic profiling of developmental changes in bovine testis by lectin histochemistry and further analysis of the most prominent alteration on the level of the glycoproteome by lectin blotting and lectin affinity chromatography. *Histol. Histopathol.* **19**, 1043–1060.
7. Thaysen-Andersen, M., Thogersen, I. B., Lademann, U., Offenberg, H., Giessing, A. M. B., Enghild, J. J., Nielsen, H. J., Brunner, N., and Hojrup, P. (2008) Investigating the biomarker potential of glycoproteins using comparative glycoprofiling – application to tissue inhibitor of metalloproteinases-1. *Biochim. Biophys. Acta* **1784**, 455–463.
8. Uchiyama, N., Kuno, A., Tateno, H., Kubo, Y., Mizuno, M., Noguchi, M., and Hirabayashi, J. (2008) Optimization of evanescent-field fluorescence-assisted lectin microarray for high-sensitivity detection of monovalent oligosaccharides and glycoproteins. *Proteomics* **8**, 3042–3050.
9. Kuno, A., Uchiyama, N., Koseki-Kuno, S., Ebe, Y., Takashima, S., Yamada, M., and Hirabayashi, J. (2005) Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling. *Nat. Methods* **2**, 851–856.
10. Patwa, T. H., Zhao, J., Anderson, M. A., Simeone, D. M., and Lubman, D. M. (2006) Screening of glycosylation patterns in serum using natural glycoprotein microarrays and multi-lectin fluorescence detection. *Anal. Chem.* **78**, 6411–6421.
11. An, H. J., Miyamoto, S., Lancaster, K. S., Kirmiz, C., Li, B. S., Lam, K. S., Leiserowitz, G. S., and Lebrilla, C. B. (2006) Profiling of glycans in serum for the discovery of potential biomarkers for ovarian cancer. *J. Proteome Res.* **5**, 1626–1635.
12. Kirmiz, C., Li, B. S., An, H. J., Clowers, B. H., Chew, H. K., Lam, K. S., Ferrige, A., Alecio, R., Borowsky, A. D., Sulaimon, S., Lebrilla, C. B., and Miyamoto, S. (2007) A serum glycomics approach to breast cancer biomarkers. *Mol. Cell. Proteomics* **6**, 43–55.
13. Kyselova, Z., Mechref, Y., Al Bataineh, M. M., Dobrolecki, L. E., Hickey, R. J., Vinson, J., Sweeney, C. J., and Novotny, M. V. (2007) Alterations in the serum glycome due to metastatic prostate cancer. *J. Proteome Res.* **6**, 1822–1832.
14. de Leoz, M. L. A., An, H. J., Kronewitter, S., Kim, J., Beecroft, S., Vinall, R., Miyamoto, S., de Vere White, R., Lam, K. S., and Lebrilla, C. B. (2008) Glycomic approach for potential biomarkers on prostate cancer: profiling of N-linked glycans in human sera and pRNS cell lines. *Dis. Markers: Glycobiol.* **25**, 243–258.
15. Kita, Y., Miura, Y., Furukawa, J., Nakano, M., Shinohara, Y., Ohno, M., Takimoto, A., and Nishimura, S. (2007) Quantitative glycomics of human whole serum glycoproteins based on the standardized protocol for liberating N-glycans. *Mol. Cell. Proteomics* **6**, 1437–1445.
16. Morelle, W., Flahaut, C., Michalski, J. C., Louvet, A., Mathurin, P., and Klein, A. (2006) Mass spectrometric approach for screening modifications of total serum N-glycome in human diseases: application to cirrhosis. *Glycobiology* **16**, 281–293.
17. Kam, R. K. T., Poon, T. C. W., Chan, H. L. Y., Wong, N., Hui, A. Y., and Sung, J. J. Y. (2007) High-throughput quantitative profiling of serum N-glycome by MALDI-TOF mass spectrometry and N-glycomic fingerprint of liver fibrosis. *Clin. Chem.* **53**, 1254–1263.
18. Harvey, D. J., Royle, L., Radcliffe, C. M., Rudd, P. M., and Dwek, R. A. (2008) Structural and quantitative analysis of N-linked glycans by matrix-assisted laser desorption ionization and negative ion nanospray mass spectrometry. *Anal. Biochem.* **376**, 44–60.
19. Isailovic, D., Kurulugama, R. T., Plasencia, M. D., Stokes, S. T., Kyselova, Z., Goldman, R., Mechref, Y., Novotny, M. V., and Clemmer, D. E. (2008) Profiling of human serum glycans associated with liver cancer and cirrhosis by IMS-MS. *J. Proteome Res.* **7**, 1109–1117.
20. Zhao, J., Qiu, W. L., Simeone, D. M., and Lubman, D. M. (2007) N-linked glycosylation profiling of pancreatic cancer serum using capillary liquid phase separation coupled with mass spectrometric analysis. *J. Proteome Res.* **6**, 1126–1138.
21. Lebrilla, C. B. and An, H. J. (2009) The prospects of glycan biomarkers for the diagnosis of diseases. *Mol. Biosyst.* **5**, 17–20.
22. Leiserowitz, G. S., Lebrilla, C., Miyamoto, S., An, H. J., Duong, H., Kirmiz, C., Ll, B., Liu, H., and Lam, K. S. (2008) Glycomics analysis of serum: a potential new biomarker

for ovarian cancer? *Int. J. Gynecol. Cancer* **18**, 470–475.
23. Li, B. S., An, H. J., Kirmiz, C., Lebrilla, C. B., Lam, K. S., and Miyamoto, S. (2008) Glycoproteomic analyses of ovarian cancer cell lines and sera from ovarian cancer patients show distinct glycosylation changes in individual proteins. *J. Proteome Res.* **7**, 3776–3788.
24. Lebrilla, C. B. and An, H. J. (2008) The prospects of glycan biomarkers for the diagnosis of diseases. *Mol. Biosyst.* **5**, 17–20.
25. Greiner, M., Pfeiffer, D., and Smith, R. D. (2000) Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. *Prev. Vet. Med.* **45**, 23–41.
26. Barkauskas, D. A., An, H. J., Kronewitter, S. R., de Leoz, L. M., Chew, H. K., de Vere White, R. W., Leiserowitz, G. S., Miyamoto, S., Lebrilla, C. B., and Rocke, D. M. (2009) Detecting glycan cancer biomarkers in serum samples using MALDI FT-ICR mass spectrometry data. *Bioinformatics*, **25**, 251–257.
27. Hopkins, A. M., Miller, C. J., Connolly, A. J., Genovese, C., Nichol, R. C., and Wasserman, L. (2002) A new source detection algorithm using the false-discovery rate. *Astron. J.* **123**, 1086–1094.