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Alterations in the Serum Glycome due to Metastatic Prostate Cancer

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Abstract

Glycomic profiles derived from human blood sera of 10 healthy males were compared to those from 24 prostate cancer patients. The profiles were acquired using MALDI-MS of permethylated N-glycans released from 10-μl sample aliquots. Quantitative permethylation was attained using solid-phase permethylation. Principal component analysis of the glycomic profiles revealed significant differences among the two sets, allowing their distinct clustering. The first principal component distinguished the 24 prostate cancer patients from the healthy individuals. It was determined that fucosylation of glycan structures is generally higher in cancer samples (ANOVA test *p*-value of 0.0006). Although more than 50 N-glycan structures were determined, twelve glycan structures, of which six were fucosylated, were significantly different between the two sample sets. Significant differences were confirmed through two independent statistical tests (ANOVA and ROC analyses). Ten of these structures had a significantly higher relative intensities in the case of the cancer samples, while the other two were less abundant in the cancer samples. All 12 structures were statistically significant, as suggested by their very low ANOVA scores (< 0.001) and ROC analysis, with area under the curve values close to 1 or zero. Accordingly, these structures can be considered as cancer–specific glycans and potential prostate cancer biomarkers. Therefore, serum glycomic profiling appears worthy of further investigation to define its role in cancer early detection and prognostication.

Keywords

prostate cancer; glycomics; human blood serum; biomarkers; MALDI-TOF; mass spectrometry

INTRODUCTION

Prostate cancer (PCa) is the most common male cancer in America, accounting to 33% of all cancer cases. The American Cancer Society (ACS) estimates that about 234,460 new cases will be diagnosed during 2006 in the United States of which 27,350 cases will be fatal, making it second only to lung cancer on the list of the leading causes of male cancer deaths. [1] While the exact causes of prostate cancer are under investigation, several risk factors have been associated with the disease, including age (2 out of 3 men with prostate cancer are older than 65), race (African-American men are twice as likely to die of PCa,

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while it occurs less often among Asian men), family history, and diet. As with any other cancer, early detection and treatment of clinically relevant prostate cancer has the potential to decrease the morbidity and mortality of this disease.

Currently, serum prostate-specific antigen (PSA) is used clinically to screen for prostate carcinoma.[2] A PSA level greater than 4 ng/mL is considered to be indicative of potential prostate cancer. However, the specificity of this test is far from being satisfactory.[3] For example, there is only 25% likelihood of having prostate cancer, if the PSA values are between 4 and 10 ng/mL. Conversely, prostate cancer may be present even when the PSA concentration is below 4 ng/ml. Biopsy or a digital rectal exam (DRE) are recommended for further diagnostic specification.[4] The lack of specificity of the PSA test is partially due to the observed increase in this protein with other prostatic pathologies such as benign prostatic hyperplasia (BPH) or prostatitis (prostate gland infection or inflammation). PSA also increases with age and infections of the prostate. Accordingly, several approaches have been developed recently to enhance PSA specificity, such as the PSA index (defined as the free PSA expressed as a percentage of the total PSA), PSA density, PSA velocity, complex PSA/total PSA ratio, or determination of different PSA forms.[5] Despite all these attempts, PSA levels still do not allow a clear differentiation between PCa and benign prostate diseases. Therefore, more specific PCa biomarkers are urgently required to facilitate early detection of clinically relevant prostate cancer.

Over the past several years, the potential of proteomic analyses in biomedical science and their clinical applications have been explored. Different research activities have focused on the utility of the proteomic approaches to define PCa biomarkers.[6-9] Owing to these research activities, 14 proteins were identified as potential serologic PCa biomarkers with different specificity and variable selectivity,[10] of which 11 proteins featured N-glycosylation motifs and potential O-glycosylation sites. In contrast, glycomic measurements have thus far been limited due to methodological problems.

For a number of years, aberrant glycosylation has been implicated in all types of cancer, where many glycosyl epitops are known to constitute tumor-associated antigens.[11-13] Also, it has been shown that molecular changes in glycosylation could be associated with the signaling pathways of the malignant transformation of cells.[14] Since the correlation between certain structures of glycans and a clinical prognosis in cancer were first suggested a decade ago,[15-16] the interest in structural studies of glycans and other related molecules on cellular surfaces has increased substantially. It was demonstrated recently that the extent of sialylation of the glycans originating from PSA, when isolated from normal cells, was lower than that of the cancer cells, while the overall fucosylation of glycan structures was higher in cancer cells relative to normal.[17-19] A difference between glycan structures of the PSA isolated from the sera of prostate cancer patients and those from the patients with benign prostate hyperplasia was also demonstrated.[20]

Due to the limited specificity of the PSA routine test, it is desirable to develop additional, more diagnostically informative procedures for the blood analysis in cancer cases. The previously recognized association of cancerous cells with unusual glycosylation of their surface proteins and their subsequent shedding into the circulating fluids provides one such opportunity. In order to recognize reliably the relevant glycan structures which may be associated with pathologically distinct glycoconjugates, it is essential to display many candidate glycans in a compound profile at sufficiently high sensitivity. Fortuitously, modern biomolecular mass spectrometry (MS) provides such an opportunity, as it has recently become one of the choice methodologies in glycomic investigations,[21-24] in general.

The uses of MS in the femtomole range and below have become common in the proteomic studies for several years, while they have been less frequent in carbohydrate analysis.[25-29] Whereas human blood has been known as a rich source of structurally and functionally diverse glycoproteins, their numerous glycosylation sites and complex microheterogeneities necessitate highly sensitive MS techniques. Nevertheless, the usefulness of MALDI (matrix-assisted laser desorption-ionization) MS has recently been appreciated in a complex profiling for breast cancer[21-23] and ovarian cancer studies.[24] In the procedure reported here, the quantitative nature of MS profiling and its sensitivity have been significantly strengthened by the application of a permethylation procedure [30] which allows a simultaneous quantitative recording of both neutral and acidic glycans. The overall procedure is sensitive, relatively rapid, and reliable.

A simple methodological approach involving MS measurements has been devised here, in which a small volumetric aliquot (10 μL serum) provides sufficient amounts to display the N-linked glycan structures in a profile (or a "glycomic map") that seems statistically indicative of a presence of prostate cancer. The study has involved recording and comparisons of the glycomic profiles from the blood sera of 10 healthy individuals and 24 PCa patients. This procedure does not implicate any particular serum glycoproteins, but rather deals with the sum, or a "glycan reservoir", of protein-free molecular entities whose total profile (qualitatively or quantitatively) becomes diagnostically indicative.

MATERIALS AND METHODS

Materials

The endoglycosidase, Peptide-N-Glycosidase F (PNGase F; EC 3.5.1.52), isolated from *E. coli*, used for deglycosylation, was obtained from Cape Cod Company (East Falmouth, MA). Trifluoroethanol (TFE), 2,5-dihydroxybenzoic acid (DHB), and sodium hydroxide were purchased from Aldrich (Milwaukee, WI). Chloroform, iodomethane and sodium chloride were received from EM Science (Gibbstown, NJ). Dithiothreitol (DTT) and iodoacetamide (IAA) were the products of Bio-Rad Laboratories (Hercules, CA). Ammonium bicarbonate was received from Mallinckrodt Chemical Company (Paris, KY). Acetonitrile (ACN) was purchased from Fisher Scientific (Fair Lawn, NJ). All other common chemicals of analytical-grade quality were purchased from Sigma (St. Louis, MO).

Serum samples and clinical diagnosis

Serum from clotted human male whole blood samples (healthy individuals) and serum samples from patients with documented metastatic prostate cancer were collected using an IUPUI IRB approved clinical trial conducted through the Hoosier Oncology Group. Venous blood samples were taken in the morning's fasting state, being collected with the minimal stasis in evacuated tubes. After at least 30 min, but within 2 h, the tubes were centrifuged at 20 °C for 12 min at 1200 *g*, and the sera were stored frozen in plastic vials at −80 °C until the time of the consecutive analyses.

The glycomic profiles were generated for the samples derived from men in the following two categories: (1) non-diseased healthy individuals; and (2) men with confirmed PCa who were undergoing androgen-deprivation therapy (ADT). In this study, the serum for the latter was obtained at the time of starting ADT. As such, the patients enrolled on this study had a documented disease burden of PCa. A detailed characterization of the patients with their clinical diagnosis can be found as Supplementary Table 1.

Reduction, alkylation and release of N-glycans from glycoproteins

Human serum samples were reduced and alkylated as previously described.[31] Briefly, a 10-μl aliquot of human blood serum was lyophilized and then resuspended in 25 μl of 25 mM ammonium bicarbonate, 25 μl of TFE and 2.5 μl of 200mM DTT prior to incubation at 60 °C for 45 min. A 10-μl aliquot of 200 mM IAA was then added and allowed to react at room temperature for 1 h in the dark. Subsequently, a 2.5-μl aliquot of DTT was added to react with the excess IAA. Next, the reaction mixture was diluted with 300 μl of water and 100 μl of ammonium bicarbonate stock solution to adjust pH to 7.5 - 8.0, as suitable for the enzymatic release of N-glycans using PNGase F according to our previously published procedure.[27] A 5 mU aliquot of PNGase F was added to the reaction mixture and susbsequently incubated overnight (18-22 h) at 37 °C.

Solid-phase extraction of enzymatically released N-glycans

The volume of enzymatically released glycans was adjusted to 1 ml by adding deionized water. Samples were then applied to both C18 Sep-Pak® cartridges (Waters, Milford, MA) and activated charcoal cartridges (Harvard Apparatus, Holliston, MA). The use of C18 Sep-Pak® cartridges is necessary to isolate the glycans from peptides and proteins, which would otherwise interfere with trapping on the activated charcoal cartridges. The reaction mixture was first applied to C18 Sep-Pak® cartridge that had been preconditioned with ethanol and deionized water according to the manufacturer's recommendation. The reaction mixture was circulated through the C18 Sep-Pak® cartridge 5 times prior to washing with water. Peptides and O-linked glycopeptides were retained on the C18 Sep-Pak® cartridge, while the released glycans were collected as eluents. Next, the C18 Sep-Pak® cartridge was washed with 1 ml of deionized water. The combined eluents containing the released N-glycans were then passed over activated charcoal microcolumns. The columns were pre-conditioned with 1 ml of ACN and 1 ml of 0.1% trifluroacetic acid (TFA) aqueous solution, as recommended by the manufacturer. After applying the sample, the microcolumn was washed with 1 ml of 0.1% TFA aqueous solution. The samples were then eluted with a 1-ml aliquot of 50% ACN aqueous solution containing 0.1% TFA. Finally, the purified glycans were evaporated to dryness using vacuum CentriVap Concentrator (Labconco Corporation, Kansas City, MO) prior to solid-phase permethytion.

Solid-phase permethylation

Permethylation of enzymatically released and solid-phase purified N-glycans was accomplished utilizing our recently published solid-phase permethylation technique.[30] This approach involves packing of sodium hydroxide beads in peek tubes (1 mm i.d.; Polymicro Technologies, Phoenix, AZ), permitting complete derivatization. Tubes, nuts and ferrules from Upchurch Scientific (Oak Harbor, WA) were employed for assembling the sodium hydroxide capillary reactor. Sodium hydroxide powder was suspended in ACN for packing. A 100-μl syringe from Hamilton (Reno, NV) and a syringe pump from KD Scientific, Inc. (Holliston, MA) were employed for introducing the sample into the reactor. Sodium hydroxide reactor was first conditioned with 60 μl of dimethyl sulfoxide (DMSO) at a 5 μl/min flow rate. Samples were resuspended in DMSO and mixed with methyl iodide solution containing traces of deionized water. Typically, released and purified N-glycans were resuspended in a 50-μl aliquot of DMSO, to which 0.3 μl of water and 22 μl methyl iodide were added. This permethylation procedure has been shown to minimize oxidative degradation, peeling reactions as well as to avoid the need of excessive clean-up.[30] Sample was infused through the reactor at a slow flow rate of 2 μl/min, as previously described.[30] The reactor was then washed with 230 μl ACN (flow rate: 5 μl/min). All eluents were combined, and the permethylated N-glycans were finally extracted using 200 μl chloroform and washed repeatedly (3 times) with 200 μl of water prior to drying.

MALDI-TOF MS instrumentation

Permethylated glycans were resuspended in 2 μl of (50:50) methanol:water solution. A 0.5-μl aliquot of the sample was then spotted directly on the MALDI plate and mixed with the equal volume of DHB-matrix prepared by suspending 10 mg of DHB in 1 ml of (50:50) water:methanol solution, containing 1 mM sodium acetate. The inclusion of sodium acetate is to promote a nearly complete sodium adduct formation in MALDI-MS. The MALDI plate was then dried under vacuum to ensure uniform crystallization. Mass spectra were acquired using the Applied Biosystems 4800 MALDI TOF/TOF Analyzer (Applied Biosystems Inc., Framingham, MA). This instrument is equipped with Nd:YAG laser with 355-nm wavelength. MALDI-spectra were recorded solely in the positive-ion mode, since permethylation eliminates the negative charge normally associated with sialylated glycans.

Data evaluation

The obtained MALDI-MS data were further processed using DataExplorer 4.0 (Applied Biosystems, Framingham, MA) to generate ASCII files listing *m/z* values and intensities. An in-house developed software tool (PeakCalc 2.0) was then used to extract the intensities of N-glycans. Principal component analysis (PCA) was performed using MarkerView (ABI, Framingham, MA), allowing the visualization of multivariate information. Supervised PCA methods were employed, using a prior knowledge of the sample groups as healthy *vs.* diseased. MS data were weighted using the base-e logarithm of the peak intensities. The peak intensities were also scaled using pareto option in which each value is subtracted by the average and divided by the square root of the standard deviation. This option is suitable for MS data, since it prevents intense peaks from completely dominating the PCA process, thus allowing any peak with good signal-to-noise ratio to contribute.

We also used Receiver Operating Characteristics (ROC) curve analysis using AccuROC 2.5 software for Windows (Accumetric Corporation, Montreal, Canada) to assess the sensitivity and selectivity of the potential diagnostic variables. ROC curve is defined as a plot of test sensitivity, in its y-axis versus its specificity or false positive rate as the x-axis. This type of statistical analysis is an effective method of evaluating the quality or performance of diagnostic tests, and has been widely used in radiology to evaluate performance of many radiological tests.[32]

Our data were also statistically analyzed using a single factor Analysis of Variance (ANOVA) test. A difference between the two groups of data was considered statistically significant when *p*-values were less then 0.05, suggesting a less than 5% probability that the difference between the two groups is statistically not significant.

The range of values throughout this study was expressed as a standard error of mean (SEM) value, which accounts for a sample size. Standard deviation is the most common measure of statistical dispersion, measuring how spread-out the values in a data set appear (e.g., due to limitations in measurement reproducibility). However, when working with biological samples, any observed variation observed might be intrinsic to the phenomenon that distinct members of a population differ greatly (biochemical individuality). Consequently, the standard error (SE), or SEM, signifies an estimate of the standard deviation of the sampling distribution of means, based on the data from one or more random samples. SEM then accounts for the number of real samples, implicating their biodiversity in the evaluation process.

RESULTS

Glycomic profiles derived from human sera

The comparative glycomic approach utilized here allowed quantitative distinction between the glycan structures derived from the sera of healthy individuals and metastatic PCa patients. The profiles of permethylated N-glycans derived from 10 μl of serum volumes were recorded for the *m/z* range of 1500-5000 using MALDI-MS. The profiles generally appeared as different between the two sample sets. Representative mirror spectra for the N-glycans derived from a healthy individual and a PCa patient are seen in Figure 1. Structural assignment of the different glycans depicted in Figure 1 were based on both enzymatic sequencing using exoglycosidases and tandem MS.

Recently, the repeatability of mass spectrometry for glycomic analysis has been addressed in a pilot study conducted by the HUPO HGPI (Human Proteome Organisation Human Disease Glycomics/Proteome Initiative) and included 20 different laboratories including ours.[33] The study involved the mass spectrometry analysis of N-linked glycans of a control sample in 20 laboratories, and the chromatographic and mass spectrometric analysis results were evaluated. The study concluded that in general, MALDI/TOF MS of permethylated oligosaccharide mixtures carried out in six laboratories yielded good quantitation, and the results can be correlated to those of chromatography of reductive amination derivatives. The study also revealed the high repeatability and reproducibility of MS analysis of permethylated glycans.

Principal component analysis of measured spectra

For an informative statistical analysis of the acquired glycomic profiles, the PCA procedure was employed, as is commonly used in microarray research for cluster analysis. PCA is designed to capture a variance in the given data sets in terms of their principal components, meaning a set of variables which defines a projection encapsulating the maximum amount of variation in a dataset. It is orthogonal (and, therefore, uncorrelated) to the previous principal component.[34,35] PCA is a chemometric tool which is commonly employed to establish the differences among sample sets.

A plot of the scores of principal component one and two for the healthy and prostate cancer samples is illustrated in Figure 2. The two sets of samples received a distinguishable first principal component (PC1) scores. Thus, the healthy samples received positive PC1 scores, while the cancer samples attained negative PC1 scores. Consequently, the two sets clustered in a manner allowing the distinction between the glycomic profiles derived from healthy individuals and those derived from PCa patients (Figure 2). This aspect of PCA suggests that there is a potential of using glycomic profiling as a diagnostic tool that can discriminate between diseased and non-diseased states and could potentially be capable of detecting an early stage of cancer.

Changes in intensities for particular structures

Classification of N-glycans into structural groups could be another means to determine major differences in the glycosylation patterns. The relative intensities of different N-glycans detected in both healthy and cancer samples were compared according to the glycan types, including the usual classification into the high-mannose, complex biantennary (with or without fucosylation), complex triantennary (with or without fucosylation), complex tetraantennary (with or without fucosylation), and, hybrid, fucosylated and sialylated types (Table 1).

A decrease in the relative intensities of high-mannose and complex biantennary structures and the concomitant increase in the fucosylated complex biantennary, complex tri- and tetraantennary N-glycans (both fucosylated and non-fucosylated) was consistent among all samples, exhibiting the overall ANOVA test *p*-values lower than 0.007. An overall decrease in smaller N-glycans (*m/z* 1500-2700) was also observed for the cancer samples, being consistent with an overall increase of the larger N-glycans (*m/z* 2700-5000). The alteration in glycosylation has been previously correlated to tumor progression.[11-13,21,36] The differences observed in our work among the high-mannose, complex biantennary, complex triantennary (fucosylated and non-fucosylated), and complex tretra-antennary (fucosylated and non-fucosylated) show ANOVA test *p*-values better than 0.01, indicating there is a less than 1% probability that this difference is due to a chance alone. An increase in the overall fucosylation in the case of the cancer samples thus appears statistically significant, as suggested by the ANOVA test *p*-value of 0.0006. A correlation between the increase in fucosylation and cancer progression has also been previously noted through different means[37] in cancer tissues.

ROC and ANOVA analyses

A statistical evaluation of changes in the intensities associated with all N-glycans that were observed in the MALDI mass spectrum was further performed to validate the aforementioned differences for the individual glycans, thus testing their potential use for cancer diagnosis. Such statistical evaluations were performed using two independent approaches: ANOVA and ROC curve analyses. ROC analysis is used in the test situations where the diagnostic test yields numerical results that can be compared to an independent diagnosis, confirming either the presence or absence of a disease.[38,39] The software was designed for a medical use and operates on two parameters: sensitivity and specificity. The AccuROC software evaluates the area under the ROC curve (AUC), which numerically describes performance of a particular analysis.[40] AUC is a combined measure of sensitivity and specificity and thus the overall performance of a diagnostic test, which can be interpreted as the average value of sensitivity for all possible values of specificity. It can take on any value between 0 and 1, since both x and y axes can have values in that range. The closer AUC is to 1, the better the overall diagnostic performance of this test, while a test with an AUC value of 1 is the one that is perfectly accurate. A test is considered to be highly accurate for the AUC values of 0.9 or higher, while a moderately accurate test demonstrates AUC value between 0.7-0.89. A test with AUC lower than 0.7 is considered to be inaccurate.[38,39]

Some illustrative examples of the ROC curves for different N-glycans are shown in Figure 3, while using the three criteria of evaluation that are commonly observed with ROC test AUC values. The N-glycan with an *m/z* value of 2285 demonstrates an AUC value of 0.94 ± 0.04 (Figure 3a), thus making this particular structure highly predictive of the disease state. The AUC value of *m/z* 2431 is 0.75 ± 0.08 (Figure 3b), suggesting a moderate accuracy. An AUC value of 0.55 ± 0.09, calculated for the N-glycan with mass of 2605 is suggesting a lack of cancer specificity for this structure (Figure 3c). Generally, the AUC values are believed to reflect the trends in the relative intensity changes of these glycan structures from a healthy, physiological state towards the diseased state. An AUC value over 0.5, and approaching 1, suggests both an increase in the accuracy and reliability of the test and an increasing trend in the relative intensities of the structures from a healthy to the diseased state. An opposite trend is observed when the AUC values are below 0.5 and approaching zero. For example, the AUC values for the glycan structures corresponding to *m/z* values 2676 and 2792 were 0.05 ± 0.04 and 0.05 ± 0.03, respectively. A decrease in the relative intensity of these two structures were observed in all cancer samples relative to the healthy

ones. The ROC test AUC values were calculated here for all different glycan structures, as summarized in Supplementary Table 2.

The changes in relative intensities were also evaluated using a single-factor ANOVA test. In this case, a statistically significant difference in the relative intensity of a glycan structure between cancer and healthy samples has been associated with a low *p*-value. Generally, a change associated with a *p*-value lower than 0.05 is considered to be significant, suggesting a 5% probability that the difference is not valid. A list of the ANOVA test *p*-values for all glycan structures was compiled, as summarized in Supplementary Table 2.

The AUC values and their corresponding ANOVA test *p*-values for the glycan structures with a moderate to highly accurate ROC analysis are listed in Table 2. Twelve of the listed N-glycans, which are highlighted in Table 2, have both ROC analysis AUC values reflecting high accuracy and *p*-values lower than 0.001. Spectra for six of the twelve structures reflecting the changes in the relative intensities between healthy and cancer samples are depicted in Figure 4, while a bar graph illustrating the overall differences for all samples is shown in Figure 5. Two of the twelve structures demonstrated a decrease in their relative intensity for all prostate cancer samples, while the other 10 demonstrated an increase as a result of cancer progression. Moreover, nine of the twelve N-glycans were sialylated to a different degree (mono-, di-, and trisialylated structures), while six were fucosylated.

Thus far, the utility of selected N-glycan structures as potential tumor biomarkers for identifying the presence of prostate cancer is strongly suggested through their significant relative differences for twelve glycan structures in the samples from the metastatic prostate patients. Moreover, these findings were confirmed by two independent statistical approaches which are validated well in biomedical research.

DISCUSSION

The analytical approach discussed here utilizes only a 10-μl aliquot of unfractionated human serum to generate glycomic profiles through solid-phase permethylation and MALDI-MS. Although the demonstrated data provide some insight into the mechanisms of altered glycosylation and point to the structural changes of glycoproteins at the onset and during the course of cancer development, no information pertaining to the proteins which endured such glycosylation changes has been obtained. While this task still awaits completion, the potential of employing the glycomic approach as a diagnostic and prognostic tool has been demonstrated.

The differences in relative intensities of over 50 N-glycans were measured in this work. Such relative intensity changes appear in accordance with the presence of disease. Such observations are also in agreement with the general knowledge that unusual glycosylation is associated with the presence of cancer.[11-13]

As demonstrated in this study, the glycomic profiles feature a significant increase in total fucosylation in correlation with presence of malignancy. This is in agreement with the previous biochemical/oncological studies using other approaches.[35, 40-45] Six of the twelve glycan structures, demonstrating a most significant increase from healthy to prostate cancer state in this study, were fucosylated. This correlates with a recent report that alpha1,2-L-fucosyltranferase exhibits an increased activity in prostate carcinoma LNPaC cells.[41] Moreover, the recent results from different types of cancer, such as pancreatic cancer,[42] colorectal cancer,[43] human leucocyte cancer,[44] hepatocarcinomas,[45] and renal carcinomas,[46] also suggest that malignancy is associated with increased fucosylation. Obviously, there is a biochemical rationale for using glycomic MS profiles as a future diagnostic and/or prognostic tool and cancer detection through blood analysis.

An additional analytical asset of our procedure is that both sialylated and non-sialylated structures can be concurrently analyzed due to the use of solid-phase permethylation, thus permitting structural correlations between neutral and sialylated glycan structures. Thus far, no significant sialylation alterations for N-glycans were observed in this study. Although an impaired sialylation pattern has been suggested in different types of cancer[19,47-49] through previous studies, our results do not implicate its importance for N-glycans. This does not rule out its significance in association with O-glycans which were not investigated at this stage.

The principal component analysis of the two groups of samples analyzed in this study demonstrates a very distinct clustering with a significant difference between the two groups. In fact, it has resulted in a complete separation between the two groups of data. The manner in which the data clustered suggest the possibility of using glycomic profiling from serum in the more involved studies investigating the effects of different therapies, a close follow-up of disease conditions, etc. Future studies are needed to investigate these possibilities.

CONCLUSIONS

The diagnostic potential of MS-based glycomic profiles has been demonstrated. A 10-μl volume of human serum is sufficient for typical analyses. Differential profiles from healthy individuals and prostate cancer patients were adequately treated through statistical tools such as PCA, ROC and ANOVA. All statistical analyses confirmed differences in the N-glycosylation patterns of healthy subjects *vs.* prostate cancer patients. The bioanalytical information acquired here provides some insight into the mechanisms of aberrant glycosylation and point to the structural changes of glycoproteins. These methodologies may form the basis for development of new pre-screening method to aid early prostate cancer detection through the determination of glycan-specific biomarkers. Such highly indicative biomarker candidates may also be able to identify patients destined to have a short versus a long remission from ADT. Further research is, therefore, required to determine whether serum glycomic profiling can be used as a screening and/or a prognostic tool.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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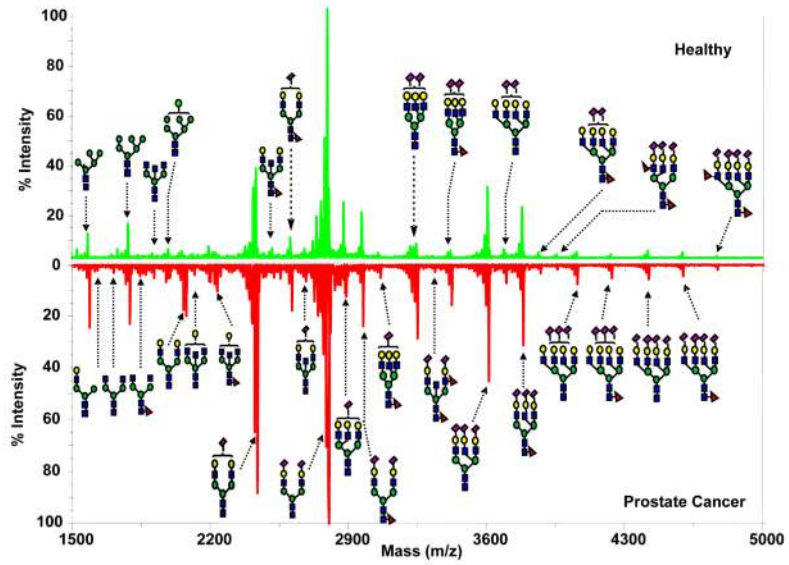

Figure 1.
MALDI mirror spectra of permethylated N-glycans derived from human blood serum of a healthy individual *vs.* a prostate cancer patient. Symbols: ■, N-acetylglucosamine; ●, mannose; ○, galactose; ▲, fucose; ◆, N-acetylneuraminic acid.

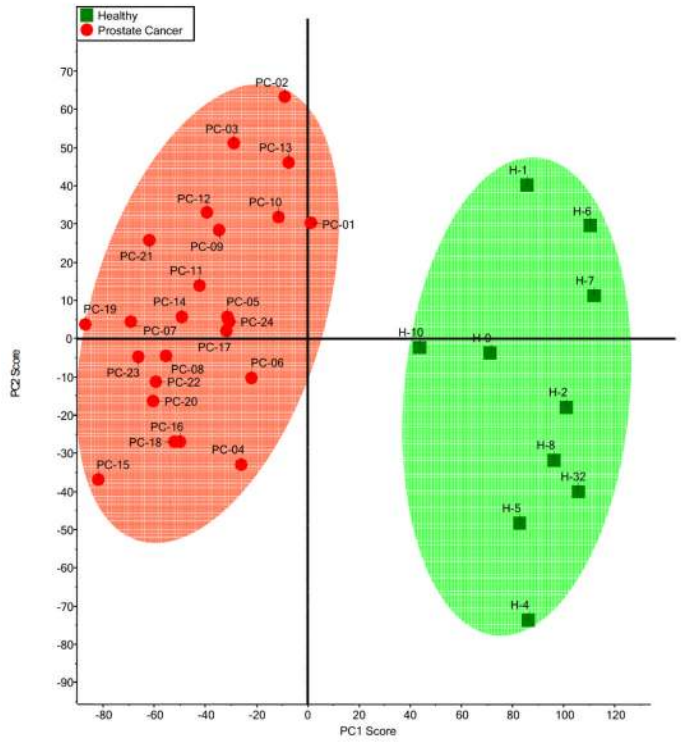

Figure 2.

Principal component analysis (PCA) scores plot for mass spectra of glycans derived from blood sera of healthy individuals (n=10) and prostate cancer patients (n=24).

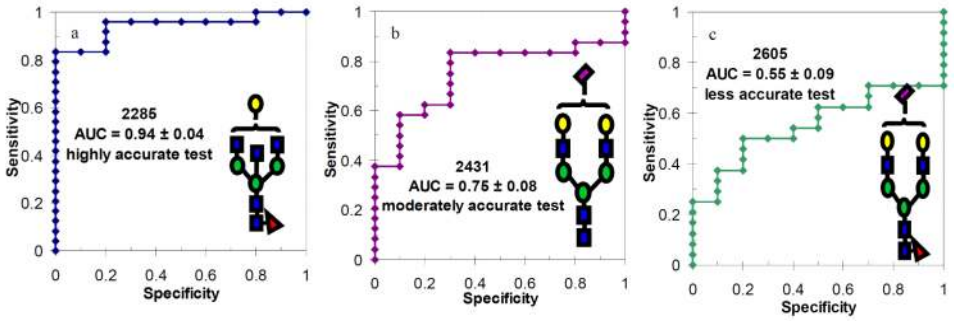

Figure 3.
Representative ROC analysis AUC plots for different glycan structures reflecting high- (a), moderate- (b), and low-accuracy (c) tests.

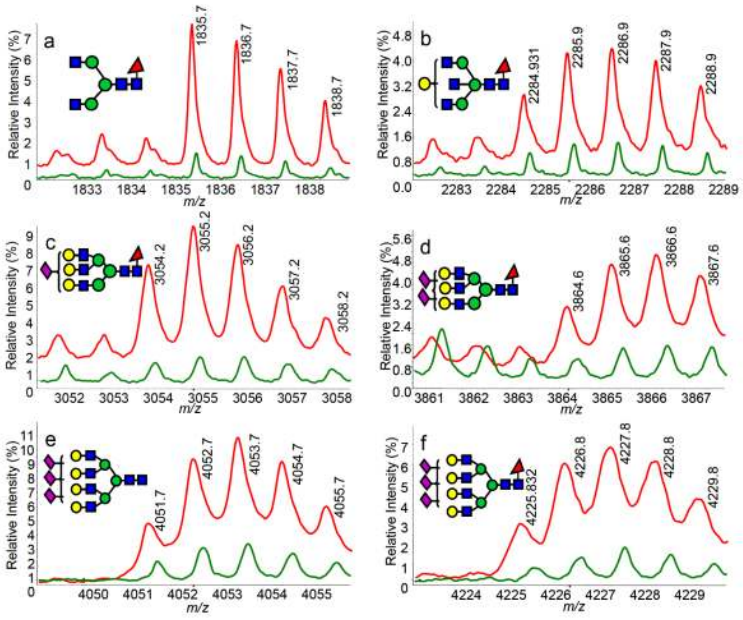

Figure 4.

MALDI mass spectra representative of the N-glycans derived from human blood serum of a healthy individual and a prostate cancer patient.

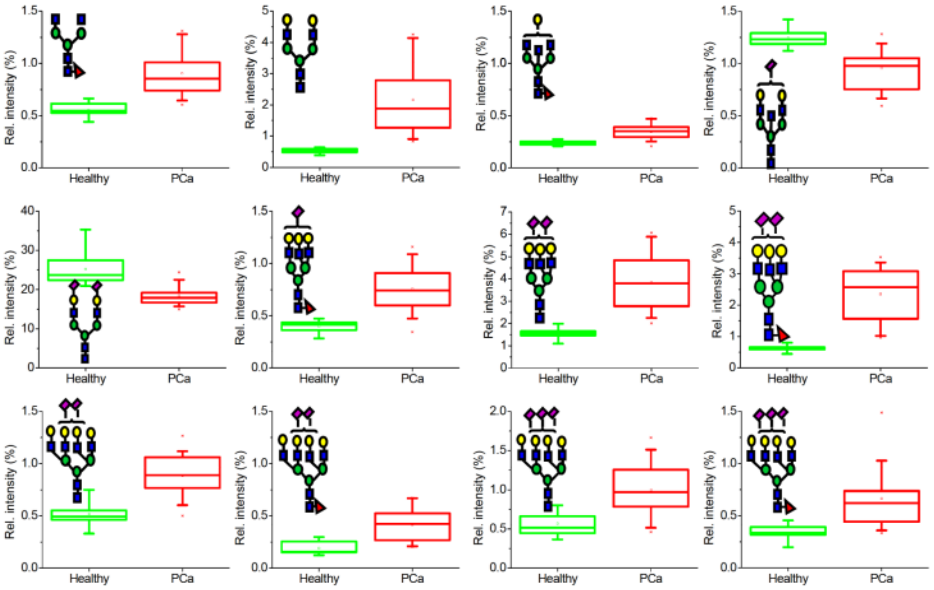

Figure 5.

Box graphs comparing the average relative intensities of all N-glycans which demonstrated highly accurate ROC analysis AUC values (0.9<AUC< 1; 0<AUC<0.1) and ANOVA test *p*-values lower than 0.001, for 10 healthy and 24 prostate cancer samples.

Table 1

Changes in the relative intensities of the types of N-glycans derived from healthy individuals and prostate cancer patients

Glycan Type	Healthy Individuals, n = 10	Prostate Cancer Patients, n = 24	ANOVA
	Relative intensity (%) ± SEM	Relative intensity (%) ± SEM	*p*-values
High mannose	16.96 ± 0.65	13.45 ± 0.79	0.007
Hybrid	2.94 ± 0.25	3.13 ± 0.17	0.5 *NS*
Complex - Bi	39.80 ± 1.65	35.54 ± 0.97	0.0005
Complex - Bi - Fuc	13.34 ± 1.22	13.42 ± 0.65	0.1 *NS*
Complex - Tri	13.92 ± 0.98	16.10 ± 0.78	0.01
Complex - Tri - Fuc	10.36 ± 0.60	13.76 ± 0.90	0.002
Complex - Tetra	1.88 ± 0.18	2.74 ± 0.18	0.005
Complex - Tetra - Fuc	1.25 ± 0.11	1.98 ± 0.20	0.007
Fucosylated	24.96 ± 1.93	29.16 ± 1.75	0.0006
Sialylated	77.36 ± 4.48	78.54 ± 3.37	0.2 *NS*

NS not significant

Table 2

Area under the curve (AUC) values from ROC analysis and p-values from ANOVA test for N-glycans derived from human blood serum of healthy individuals and prostate cancer patients. Highlighted rows are the N-glycans which received a highly accurate ROC analysis AUC values (0.9<AUC<1 or 0<AUC<0.1) and a low ANOVA test *p*-values, while others are N-glycans which received a moderately accurate ROC analysis AUC values (0.7<AUC<0.9 or 0.1<AUC<0.3) and higher ANOVA *p*-test values

N-glycan	Structure	AUC ± error	ANOVA *p*-value
1579.8		0.26 ± 0.09	0.019
1620.8		0.84 ± 0.07	0.014
1783.9		0.17 ± 0.07	0.003
1835.8		0.98 ± 0.02	4.3E-06
1865.9		0.72 ± 0.10	0.026
1906.9		0.21 ± 0.08	0.004
1989.1		0.20 ± 0.08	0.020
2070		1	1.9E-05
2081.1		0.73 ± 0.11	0.358
2111.1		0.30 ± 0.09	0.013
2192.1		0.24 ± 0.08	0.089
2244.1		0.78 ± 0.08	0.019
2285.2		0.94 ± 0.04	4.3E-05
2396.2		0.28 ± 0.10	0.031
2431.2		0.75 ± 0.09	0.059
2489.3		0.80 ± 0.09	0.010
2676.3		0.05 ± 0.04	3.0E-05
2792.4		0.05 ± 0.03	1.1E-06
2880.4		0.77 ± 0.09	0.015
3054.5		0.97 ± 0.03	5.9E-0.6
3241.6		1	7.2E-07
3385.6		0.76 ± 0.09	0.239
3415.7		1	2.9E-0.7
3690.8		0.95 ± 0.03	4.1E-06
3864.9		0.95 ± 0.04	5.8E-04
4052.0		0.89 ± 0.05	3.4E-04
4226.1		0.94 ± 0.04	9.3E-0.4