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Glycomic Analysis of Sialic Acid Linkages in Glycans Derived from Blood Serum Glycoproteins

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Abstract

A number of alterations to the normal glycomic profile have been previously described for a number of diseases and disorders, thus underscoring the medical importance of studying the glycans associated with proteins present in biological samples. An important alteration in cancer progression is an increased level of α 2,6-sialylation, which aids in increasing the metastatic potential of tumor cells. Here we report a glycomic method that selectively amidates α 2,6-linked sialic acids, while those that are α 2,3-linked undergo spontaneous lactonization. Following subsequent permethylation, MALDI-TOF-MS analysis revealed that many sialylated glycans present on glycoproteins found in blood serum featured increased levels of α 2,6 sialylation in breast cancer samples. Based on the altered ratios of α 2,3-linked to α 2,6-linked sialic acids, many of these glycans became diagnostically relevant when they did not act as such indicators when based on traditional glycomic profiling alone.

Keywords

cancer; sialic acid; permethylation; amidation; lactonization

Introduction

Breast cancer is one of the most common cancers and is responsible for 14% of all cancer-related deaths in women and 1.6% of all female deaths worldwide.¹ Presently, nearly 4.5 million women worldwide have been diagnosed with breast cancer during the last five years.¹ When detected early, breast cancer is very treatable and therefore the ability to diagnose breast cancer in its early stages is vital to significantly improving the 5-year survival rate.² However, breast cancer is often difficult to detect in its beginning, while it is commonly diagnosed in its later stages when treatment options become limited. In order to achieve an earlier detection, several protein indicators have been proposed as possible biomarkers for breast cancer. These protein markers include CA-15.3, carcinoembryonic antigen (CEA), MUC1, and human epidermal growth factor 2 (HER-2). However, most of these markers either lack the specificity or the sensitivity to accurately diagnose breast cancer so that mammography is typically used. There still exists a significant rationale to identify indicators that are more specific, sensitive, and non-invasive for the early detection of this disease. Highly-sensitive and informative analysis for biomarkers in blood serum continues to receive considerable attention.

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Supporting Information Available. This material is available free of charge via the Internet at: <http://pubs.acs.org>.

Recently, aberrant changes to the normal serum glycomic profile have been reported for a number of cancers,³⁻¹⁷ while these alterations have been suggested as a possibility for early disease detection. Interestingly, many of the protein indicators previously reported for breast cancer are glycosylated and other specific glycoproteins have been implicated as indicators of other cancers. Many of these glycoproteins, for example, CA-125 for ovarian cancer¹⁸ and prostate-specific antigen (PSA)^{8;9} for prostate cancer, feature alterations in their glycan sequences in tumor cells. For PSA derived from the serum of patients suffering from prostate cancer, a decrease in the levels of α 2,3-linked sialic acid and fucose have been reported when compared to the PSA isolated from seminal plasma.⁹ Additionally, increased levels of highly-sialylated glycans have been reported,³ indicating this carbohydrate's importance in cancer progression and its diagnosis.

Sialic acids, a family of 9-carbon containing acidic carbohydrates, often terminate the glycan structure, playing seemingly important roles in cell-cell recognition and host-pathogen interactions. These important units are found on both N- and O- linked glycans, being attached to either galactose or N-acetylglucosamine units via α 2,3- or α 2,6-linkages whose syntheses are catalyzed by specific enzymes. Increased activity levels of ST6Gal-1, the enzyme responsible for the addition of an α 2,6-linked sialic acid to galactose, has been reported for many cancers including colorectal,¹⁹ liver,²⁰ ovarian,²¹ and breast.²² Increased activity of this enzyme has been linked to poorer patient prognosis^{22;23} and its effects have been associated with decreased cell-cell interactions, increased adhesion to collagens, enhanced motility, and increased invasiveness of tumor cells.²⁴⁻²⁶ All of these factors lead to an increased metastatic ability of cancer cells and may thus be responsible for a poor prognosis. Therefore, the ability to detect and monitor changes in the sialylated glycan isomers, which are altered by enhanced activity levels of ST6Gal-1, could be an important aid in the diagnosis of cancer at an earlier stage, thus improving the patient's prognosis.

Many of the previous studies of the changes in α 2,6-linked sialylation have relied on increased binding levels of glycoproteins to *Sambucus nigra agglutinin* (SNA) lectin. These studies have been performed mainly on the extracts derived from healthy control and tumor tissues and cell lines. While this is an effective approach in its own right, it does not provide information pertaining to the specific glycan structures representing the alteration. In this study, we sought an analytical method which would be capable of measuring changes in the sialylation linkages of specific glycans present in the glycoproteins in blood serum, rather than tissue or cell extracts. In recent years, blood serum has received significant attention for glycomic analysis, presuming that the key cell-surface glycoproteins, or their fragments, that feature altered glycosylation could be released into the blood stream. Consequently, such aberrant changes associated with these biomolecules may be detectable through highly-sensitive glycomic methods.

In order to determine which particular glycans derived from glycoproteins present in blood serum may feature enhanced α 2,6 sialylation, we have adapted a glycomic method that selectively labels α 2,6-linked sialic acids,²⁷ which we followed with a subsequent solid-phase permethylation, another key methodology which has been shown to be an effective method for reaching the trace-level glycans often found in biological sources.^{28;29} Sialic acid modification was accomplished by first treating all sialylated glycans with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM),³⁰ a reagent that has been shown to be a potent activator of carboxylic acids, converting them to esters and amides. Because of the ability of the carboxylic acid moiety of α 2,3-linked sialic acids to spontaneously form cyclic, internal esters, known as lactones,³¹ only those structures possessing α 2,6-linked sialic acids are activated and subsequently modified by an appropriate nucleophile. Previously, esterification of α 2,6-linked sialic acids with methanol has been reported.³² However, due to the later permethylation, which also transforms

carboxylic acids to esters, esterification did not meet our requirements and we have thus chosen amidation with ammonium chloride to label α 2,6-linked sialic acids.

Experimental

Materials

Glycan standards with known sialic acid linkages (α 2,3-sialyllactose and α 2,6-sialyllactose), ammonium chloride, sodium hydroxide beads, β -mercaptoethanol, methyl iodide, trypsin, and bovine fetuin were all purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS) was acquired from Bio-Rad Laboratories (Hercules, CA) and Nonidet P40 was received from Roche Diagnostics (Indianapolis, IN). Graphitized carbon, C₁₈, and empty micro-spin columns along with micro-dialyzers (500 Da molecular weight cut-off) were products of Harvard Apparatus (Holliston, MA). The MALDI matrix, 2,5-dihydroxybenzoic acid (2,5-DHB), N-methylmorpholine (NMM), and 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) originated from Alfa Aesar (Ward Hill, MA) and N-Glycanase F (PNGase F) was acquired from Northstar Bioproducts (East Falmouth, MA). Water (HPLC-grade) and trifluoroacetic acid (TFAA) were obtained from EMD Chemicals, Inc. (Gibbstown, NJ), while HPLC-grade acetonitrile (ACN), chloroform, tetrahydrofuran (THF), 99.0% purity, and dimethylformamide (DMF) were purchased from Mallinckrodt Baker (Phillipsburg, NJ).

Blood Serum Samples

Blood samples were collected from cancer-free female volunteers and female patients diagnosed with late-stage breast cancer by a clinical team according to an institutional review board approved clinical trial. The samples were age-, race-, and sex- matched. Venous blood samples were acquired during the morning fasting state, with minimal stasis, into evacuated tubes. After at least 30 min, but within 2 h, the tubes were centrifuged at 20 °C for 12 min at 1200 g. The serum samples were stored at -80 °C, in plastic vials, until needed.

Preparation of 4-(4,6-dimethoxy-1,3,5-triazin-2yl)-4-methylmorpholinium chloride

The reagent used to activate the carboxylate groups of sialic acids, 4-(4,6-dimethoxy-1,3,5-triazin-2yl)-4-methylmorpholinium chloride (DMT-MM), was prepared according to the method of Kunishima *et al.*³⁰ Briefly, 2 ml (approximately 2 g) of N-methylmorpholine (NMM) and 2.9 g of 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) were added to 60 ml of THF and stirred at room temperature for 30-45 min. The resulting white precipitate was filtered, washed thoroughly with THF, and dried under vacuum.

Glycan Release and Purification

Proteins derived from human blood serum (0.5-2.5- μ l aliquots) or bovine fetuin (5 μ g aliquots) were first digested with trypsin (a 1- μ l aliquot for blood serum or a 0.25- μ l aliquot for bovine fetuin of 1 μ g/ μ l enzyme solution) for 1 hr at room temperature to prevent precipitation during subsequent steps in the procedure. Digestion was terminated and proteins were simultaneously denatured and reduced by treating the samples with a 25- μ l aliquot of 0.1%/0.1% β -mercaptoethanol/SDS solution in a 10 mM sodium phosphate buffer (pH = 7.5). The samples were incubated for 45 min at 60° C. After allowing the samples to cool to room temperature, Nonidet P40 was added to a final concentration of 1%. After allowing the solutions to equilibrate for 5-10 min, the N-linked glycans were separated from their protein backbones by incubating the samples for 18 hrs at 37 °C with 0.25 μ l of PNGase F. Next, the liberated glycans were recovered and purified from the reaction mixture through a two-step solid-phase extraction procedure. First, proteins and other hydrophobic species were removed by passing the samples through C₁₈ micro-spin columns.

The medium was first conditioned with 85%/15%/0.1% ACN/water/TFAA solution and then re-equilibrated with 95%/5%/0.1% water/ACN/TFAA solution. After sample loading, the glycans were recovered by washing the medium twice with 100 μ l of 95%/5%/0.1% water/ACN/TFAA. Graphite micro-spin columns were utilized to further purify the samples. Columns were conditioned and re-equilibrated using the same procedure used for the C₁₈ micro-spin columns. After sample loading, columns were washed twice with 200 μ l of 97%/3% water/acetonitrile solution. Neutral glycans were then eluted by the addition of two 200- μ l aliquots of 75%/25% water/acetonitrile solution and acidic glycans were subsequently eluted with two aliquots, 200 μ l each, of 75%/25%/0.1% water/acetonitrile/TFAA. The samples were next completely dried in a vacuum centrifuge.

Sialic Acid Amidation/Lactonization

Sialic acids associated with a galactose via an α 2,6 linkage were converted to amides, while those linked in an α 2,3-association spontaneously generated lactones, resulting in a mass shift in the subsequent MALDI-TOF-MS analyses. Amidation of the α 2,6-linked sialic acids was accomplished as previously described,²⁷ with minor modifications. In the present study, the glycan samples were treated with a 10- μ l aliquot of 0.5 M DMT-MM prepared in a 0.5 M ammonium chloride solution (pH = 6.5) and incubated at 60 °C for 15 hrs. The glycans were then recovered from the reaction mixture using graphite micro-spin columns, which were conditioned as previously described. However, for purification following this derivatization reaction, the medium was re-equilibrated with 90%/10% water/ACN. This solution was also used to dilute the samples to 200 μ l prior to loading them onto the spin columns and washing thoroughly the medium. The glycans were eluted, as previously described, and dried.

Static Solid-phase Permethylation

Following the amidation/lactonization reaction and clean-up, the glycans were subjected to a static solid-phase permethylation.³³ Briefly, solid-phase permethylation reactors were prepared immediately prior to their use by loading empty spin columns with sodium hydroxide beads that had been suspended in ACN. The reactors were then washed thoroughly with DMF prior to the addition of the samples. The glycan samples were solubilized in 100 μ l of a solution composed of 5%/70%/25% water/DMF/methyl iodide and allowed to react statically for 15 min. After that time, the samples were centrifuged, a second 25- μ l aliquot of methyl iodide was added, and the samples were reapplied to the reactors for an additional 15-min period. Next, the samples were again centrifuged and the reactors were washed twice with 100- μ l aliquots of acetonitrile to ensure maximum recovery. The permethylated glycans were recovered from the reaction mixture by liquid-liquid extraction with 300 μ l of chloroform and a repeated washing with 1-ml aliquots of 0.5 M NaCl and HPLC-grade water.

MALDI Mass Spectrometry

All mass-spectrometric measurements were performed on an Applied Biosystems (Applied Biosystems, Inc. Framingham, MA) 4800 MALDI-TOF/TOF mass spectrometer. The glycan samples were resolubilized in 2.5 μ l of 20%/80% methanol/water solution and a 0.5- μ l aliquot was spotted on a stainless steel MALDI target and dried. The matrix, 2,5-DHB, was prepared at a concentration of 10 mg/ml in 50%/50% methanol/water solution with 1 mM sodium acetate to ensure complete cationization and a 0.5- μ l aliquot of matrix was added to the sample spot and dried under vacuum to promote uniform crystallization. The instrument was operated in the positive-ion reflector mode and the m/z range from 2000-5000 was monitored. A total of 2000 laser shots were applied to each sample.

Data Analysis

MALDI-TOF-MS data were baseline-corrected, a noise filter was applied, and the spectra were exported as text files using DataExplorer (version 4.0), which is included with the instrument software. The normalized intensity for each glycan isomer ion was calculated from the total ion intensity for all ions of a given glycan sequence included in this study using an in-house developed software tool. Microsoft Excel was used to perform single-variable analysis of variance (ANOVA), while Origin 8 (OriginLab Corporation, Northampton, MA) was used to perform the receiver operating characteristics statistical analyses.

Results

The use of DMT-MM to modify α 2,6-linked sialic acids through esterification with methanol and lactone formation of α 2,3-linked units was already reported.³² This reaction was performed at 80 °C for 1 hr. While this is a generally effective procedure, we found that during such permethylation, the lactone is cleaved to regenerate the carboxylate, which also undergoes permethylation, resulting in an ester. Therefore, any information about the sialic acid linkages can be lost unless the more expensive isotopically-labeled reactants (either methanol or methyl iodide) become used. Consequently, we have developed a sample preparation procedure using amidation, similar to that previously reported,²⁷ that is compatible with permethylation and retains data about the linkages of the sialic acids present on a glycan.

Efficiency and Selectivity of Amidation/Lactonization

In the previous report discussing amidation of sialic acids using DMT-MM,²⁷ glycan structures with only α 2,6-linked sialic acids were studied and there was no mention of a lactone formation for α 2,3-linked sialic acids. Thus, the selectivity of the amidation/lactonization reaction was studied here to ensure that only α 2,6-linked sialic acids underwent quantitative amidation. Further, it is critical for the real-world biological samples to ensure that α 2,3-linked structures undergo complete lactonization and do not participate in any side reactions that could lead to amidation. A failure to ensure completion of these two complementary reactions could lead to ambiguities or false conclusions based on incorrect ratios of α 2,3- and α 2,6- linked isomers in the glycans derived from biological samples.

The selectivity and efficiency of the amidation/lactonization reaction with a subsequent permethylation was performed separately on 1 pmole of α 2,3-sialyllactose and α 2,6-sialyllactose, as previously described. After the amidation/lactonization reaction, the sialyllactoses were purified through dialysis against water with 500 Da molecular weight cut-off membranes for 24 hrs and then permethylated. Figure 1a and b presents the reaction schemes for α 2,3-sialyllactose and α 2,6-sialyllactose, respectively, after both derivatization reactions and demonstrate the differential labeling of these isomers. Comparing Figure 1a and b reveals a mass difference of 13 Da for these isomers. During permethylation, the two hydrogen atoms attached to the amide nitrogen of the α 2,6-sialyllactose are replaced with two methyl groups, while the carboxylate group of the α 2,3-sialyllactose is regenerated and permethylated. When the loss of 1 Da for the conversion of a carboxylic acid to an amide is considered, the mass difference between permethylated α 2,3-sialyllactose and permethylated and amidated α 2,6-sialyllactose is 13 Da, as seen in Figure 2a and b.

Figure 2a and b demonstrates that the amidation/lactonization reaction is both specific and efficient. If the α 2,3-sialyllactose isomer did not undergo a complete lactonization, and a small fraction became amidated, an ion at m/z 851 would be present. However, this ion was

not observed for α 2,3-sialyllactose, so that a spontaneous lactonization was deemed complete. Likewise, the amidation reaction was also highly efficient. For the reaction performed with α 2,6-sialyllactose, only an ion at m/z 851 was observed in the mass spectra. Therefore, the amidation approach also appears quantitative.

Reproducibility and Accuracy

The reproducibility of the entire procedure, including glycan release, sample purification, the lactonization/amidation reaction, permethylation, and mass-spectral analysis, was examined for ten 0.5- μ l aliquots of a single commercially-available human blood serum sample. (See Figure S1a-j for the MALDI-MS spectra of these analyses and the summary of these results in Table S1 in the Supplemental Data.) A total of six acidic glycans with all possible permutations of sialic-acid isomers were observed in these samples. Only those structures that showed the same isomers present in all samples were subjected to statistical analysis. Generally, glycans that produced higher normalized intensities, greater than *ca.* 1% normalized intensity, as based on glycomic profiling alone, resulted in measurements of the isomeric structures with acceptable reproducibilities. For these structures, relative standard deviations (%RSD) of approximately 10%, or less, were observed in many cases. The %RSD was significantly elevated for the lower-abundance glycans, i.e. those that resulted in a normalized intensity of less than 1% (based on glycomic analysis alone). For these analytes, % RSDs approaching 20% were common. Overall, for the six glycans observed from 0.5 μ l of human blood serum, the average %RSD for the entire experimental protocol was calculated to be 13.2 %.

The ability of the lactonization/amidation reaction to accurately reflect the correct ratios of α 2,3- and α 2,6- linked sialic acids was investigated using the N-linked glycans derived from 5 μ g of bovine fetuin. (A representative MALDI-MS spectrum of the glycan derived from bovine fetuin after the amidation/lactonization reaction and permethylation is shown as Figure S2 in the Supplemental Data.) The ratios of linkages recorded mass-spectrally were in good agreement with those generated by NMR³⁴(see Figure S3a-f in the Supplemental Data). These two studies demonstrate that the analytical procedure described here has an acceptable reproducibility and is capable of accurately mimicking the correct ratios of sialic-acid isomers and therefore may have a wider potential diagnostic use in real-world biological samples.

Analysis of Glycans Derived from Blood Serum of Cancer-free and Breast Cancer Patients

Over the past several years, much effort in the field of glycomics has been devoted to searching for more sensitive and specific indicators of pathological conditions in blood serum. It is now well-accepted that glycosylation is both cell- and tissue-specific. Thus, in pathological samples, aberrant glycosylation patterns should be identifiable from glycoproteins that may be shed from cancerous cell surfaces into the blood stream. While the exact mechanisms resulting in these changes may not be clear at this time, it may be possible that some of these alterations could be the result of increased enzymatic activity, in some cases by ST6Gal-1, the enzyme responsible for adding an α 2,6-linked sialic acid to the carbohydrate chain. Some previous findings indicate that the proteins on the cell surface undergo extensive α 2,6-linked sialylation during tumor progression²⁵ and, therefore, using this methodology, alterations in the ratios of glycan isomers possessing α 2,3-linked to α 2,6-linked sialic acids present in blood serum glycoproteins may become observable.

Previously, our laboratory reported the usefulness of glycomic profiling as a tool for the diagnosis of breast cancer.³ This work demonstrated that breast cancer progression resulted in increased normalized abundances of several larger complex-type sialylated glycans. However, many glycans, including several highly-abundant structures with associated sialic

acid units, did not result in diagnostically-relevant tests. Therefore, a goal of this project was to obtain additional diagnostic information contained in blood serum samples and possibly use the highly-abundant sialylated glycans to distinguish healthy individuals from those diagnosed with breast cancer.

Using a 2.5- μ l aliquot of blood serum, a total of 12 sialylated glycans were included in this study, as shown in Figure 3, with a representative MALDI-TOF-MS spectra after amidation/lactonization and the subsequent permethylation. In order to be subjected to further statistical analysis, the isomers identified for a particular glycan were required to be identified in all samples. From all present oligosaccharides, the dominant glycan structure identified in this study was a biantennary-disialylated glycan. Glycomic profiling of the samples demonstrated that this particular glycan was not diagnostic of breast cancer. While there appears to be a slight decrease in its normalized abundance in the breast cancer samples, as shown by the notched-box plot presented as Figure 4a, the significant overlap between the sample sets resulted in a P-value from the analysis-of-variance test (ANOVA) of 0.0693. (In this study, differences between the samples sets were considered statistically significant when the P-value was less than 0.05.)

Due to its high relative abundance and a lack of diagnostic ability based on glycomic profiling alone, this particular glycan appeared to be an ideal candidate for a further examination of potential alterations to the ratios of sialic acid linkages in breast cancer patients. Figure 4b presents the representative MALDI-TOF-MS spectra of the biantennary-disialylated glycan after lactonization/amidation and the subsequent permethylation for a cancer-free patient (top spectrum) and a patient suffering from breast cancer (bottom spectrum). (The MALDI-MS spectra for the 10 cancer-free individuals are shown in Figure S4a-j and those for the patients diagnosed with Stage IV breast cancer are given as Figure S5a-j in the Supplemental Data.) Comparing these two spectra reveals significant changes in the relative abundances of the isomers with two α 2,3-linked sialic acids (m/z 2792.4) and with two α 2,6-linked sialic acids (m/z 2818.5). In the representative spectra, the isomer present at m/z 2792.4 indicated a normalized intensity of 40.6% of the total signal associated with this set of isomers. This value was significantly decreased, while only a normalized intensity of 12.2% for the total signal was recorded, for a patient with breast cancer. Conversely, the isomer with two α 2,6-linked sialic acids (m/z 2815.5) resulted in a normalized intensity of 43.3% of the measured ion intensity associated with this glycan sequence in the cancer-free patient, and this value was increased to 68.1% in the breast cancer sample. Interestingly, the isomer bearing one of each linkage did not appear to be significantly altered between the cancer-free and pathological samples. This isomer (m/z 2805.4) was present with a normalized intensity of 16.1% of the total signal for the former, and 19.7% for the latter. The reason for this is not currently understood. The trends illustrated by the two representative spectra, shown in Figure 4b, were further validated by the notched-box plots, shown in Figure 4c, for ten cancer-free samples and ten late-stage breast cancer samples. As demonstrated by the MALDI-TOF-MS spectra in Figure 4b, the normalized intensity of the ion at m/z 2792.4 is clearly lowered in the breast cancer samples. This is statistically validated by the P-value of 1.69×10^{-4} . The isomer with one of each linkage, present at m/z 2808.5, has a complete overlap of the data sets, indicating no diagnostic value for this isomer, as further confirmed by the P-value of 0.820, indicating its poor diagnostic potential. The isomer with two α 2,6-linked sialic acids, present at m/z 2818.5, shows a substantial increase in this isomer's normalized abundance in the blood serum derived from cancer patients. The P-value for this isomer was of 1.50×10^{-4} .

A receiver-operating characteristics (ROC) test was also performed to produce an area-under-the curve (AUC) value. This test measures the sensitivity, or the true-positive rate, against the 1-specificity, or the false-positive rate. Therefore, a perfect test is one that

indicates an AUC value of either 1 (positive for the condition) or 0 (negative for the condition). Values between 0.9 and 1 or 0 and 0.1 are considered as highly-accurate tests and those between 0.8 and 0.9 or 0.1 and 0.2 are accurate tests. If an AUC value falls between 0.7 and 0.8 or 0.2 and 0.3, the test may be thought of as a moderately accurate, while an uninformative test produces an AUC value between 0.5 and 0.7 or 0.3 and 0.5. When the AUC values were calculated for the isomers of the biantennary-disialylated glycan, the isomers with both α 2,3- (m/z 2792.4) or α 2,6- (m/z 2818.5) linked structures resulted in AUC values of 0.95 and 0.05, respectively, indicating high accuracy to predict the state-of-health of an individual. The isomer with one α 2,3-linked and one α 2,6-linked sialic acid resulted in an AUC value of 0.55. Therefore, no diagnostic conclusions can be determined from this isomer. Thus, while the data obtained through glycomic profiling alone did not produce diagnostically-useful results for this glycan, it appears that an analysis of the sialic acid linkages could be diagnostically relevant.

A fucosylated analogue of the biantennary-disialylated glycan was also present with a moderate abundance in the MALDI-TOF-MS glycomic profile. Glycomic profiling and a subsequent ANOVA and ROC analysis of this glycan also demonstrated its limited capability to effectively diagnose breast cancer, as shown in Figure 5a. This figure presents the notched-box plots for the normalized intensities from the glycomic profiling of cancer-free (blue trace) and breast cancer (red trace) samples. ANOVA testing resulted in a P-value of 0.813 and ROC analysis returned an AUC value of 0.53 for this glycan. However, as demonstrated for the biantennary-disialylated glycan, analysis of the sialic acid linkages revealed enhanced α 2,6 sialylation in the breast cancer samples. While the previous example showed changes in the isomers with two α 2,3-linked or two α 2,6-linked sialic acids, the fucosylated biantennary-disialylated glycan presented statistically significant changes in the isomer with two α 2,3-linked sialic acids and the isomer with one α 2,3- and one α 2,6- linked sialic acid. These changes are depicted in Figure 5b, with a representative MALDI-TOF-MS spectra of a cancer-free patient (top spectrum) and a patient with breast cancer (bottom spectrum). For this glycan sequence, the normalized intensity for the isomer with two α 2,3-linked sialic acids (m/z 2967.1) indicates 37.3% of the total signal for this glycan sequence, for a representative cancer-free patient. This value is decreased to 29.5% for a patient with breast cancer. The other isomer exhibiting a substantial change contained one α 2,3- and one α 2,6- linked sialic acid (m/z 2980.1). In this fucosylated glycan, the abundance of this isomer is elevated in the breast cancer patient, demonstrating an average normalized intensity of 25.9% for the total signal associated with this set of isomers, while in the cancer-free subject, this isomer is present with only 16.4% of the signal. The isomer with two α 2,6-linked sialic acids for this structure does not appear to be useful in disease diagnosis. This isomer was present with approximately 45% of the total signal in both conditions. The notched-box plots resulting from the statistical analysis of ten control and ten pathological samples are presented as Figure 5c, demonstrating a lowered abundance of the isomer with both sialic acids linked α 2,3 in the breast cancer samples, while the isomer consisting of both linkage possibilities was elevated in the pathological samples. ANOVA analysis for both of these isomers resulted in the P-values less than 0.05. While it appears that the isomer with two α 2,6-linked sialic acids may be slightly down-regulated in the late-stage breast cancer patients, ANOVA testing resulted in a P-value of 0.113 for this isomer, indicating that these results were not diagnostically relevant. Further analysis by ROC resulted in moderately accurate AUC values of 0.71 and 0.21 for the isomers with P-values less than 0.05, while the isomer with both sialic acids linked α 2,6 resulted in an uninformative test, with an AUC value of 0.38. This glycan structure also demonstrates the potential ability of this method to diagnose the disease when glycomic profiling alone is unable to do so.

While the previous examples focused on the glycans that did not show statistical significance based on glycomic profiling alone, the analysis of the sialic acid linkages can also be used in tandem with glycomic profiling data for the structures that do generate diagnostic data from that profile alone. Thus, an added level of confidence in the diagnosis may be added with this additional information. This is demonstrated with a triantennary-trisialylated glycan. As shown in Figure 6a, the glycomic analysis for this glycan accurately reflects the state-of-health, based on the increased relative abundance of this glycan in the pathological samples. The ANOVA analysis of this structure resulted in a P-value of 0.0026 and the AUC value was highly accurate at 0.12. Interestingly, for this glycan, the isomers with just α 2,3- or α 2,6- linked sialic acids appeared to be altered more than those isomers possessing sialic acids with both linkages. This is shown by the representative mass spectra for a cancer-free patient (top spectrum) and a patient with late-stage breast cancer (bottom spectrum). In these spectra, the isomer with only α 2,3-linked sialic acid represented a normalized intensity of 21.3% of the total ion intensity. The analysis of sialic acid linkages from a patient with breast cancer revealed that this isomer accounted for a normalized intensity of only 9.7% of the total signal derived from these isomers. Only very minor changes were observed for the isomers composed of two α 2,3-linked and one α 2,6-linked sialic acids and one α 2,3-linked and two α 2,6-linked sialic acids. The final isomer, consisting of only α 2,6-linked sialic acids, exhibited a substantial elevation in its relative abundance in the cancer sample. This isomer represented nearly 42% of the average total intensity in the pathological state, while only 25.9% in the cancer-free sample. The changes indicated by the representative MALDI-TOF-MS spectra are validated in Figure 6c, which presents the notched-box plots for this glycan in the analysis of ten cancer-free and ten late-stage breast cancer patients. In this figure, obvious changes are observed for the glycan isomers with only α 2,3-linked and α 2,6-linked sialic acids. The remaining isomers do not appear capable of diagnosing breast cancer. A further statistical analysis by ANOVA reveals that the changes for the isomers with three α 2,3-linked and three α 2,6-linked sialic acids were statistically altered as reflected by the P-values of 0.00292 and 0.00448, respectively. However, the structure with two α 2,3- and one α 2,6- linked sialic acids was not significant, with a P-value of 0.640. Consequently, a minor alteration (approximately 2%) was not significant for this isomer. The isomer with one α 2,3-linked sialic acid and two α 2,6-linked sialic acid was also diagnostically irrelevant, as reflected by the P-value of 0.916. The AUC values further validated the ANOVA results. For the isomers with only α 2,3-linked sialic acid or α 2,6-linked sialic acids, AUC values of 0.22 and 0.85, respectively, were obtained. The isomer with one α 2,3- and two α 2,6- linked sialic acids resulted in an AUC value of 0.38, indicating that the subtle lowered abundance of this isomer was indeed insignificant. An AUC value of 0.59 was calculated for the isomer with two α 2,3- and one α 2,6- linked sialic acids, indicating the lack of diagnostic value for this particular isomer.

Discussion

Overall, 12 oligosaccharides were selected in this study while analyzing 2.5- μ l aliquots of blood sera. In general, a trend towards higher levels of α 2,6-linked sialic acids was observed in the breast cancer samples for all of the glycans included in this study. Conversely, the cancer-free subjects tended to feature increased levels of α 2,3 sialylation. These results are summarized in Table 1. These data are consistent with the previously published results for a number of other cancers, as based on an enhanced binding of proteins derived from tumor tissues and cell lines to the SNA lectin^{26;35} or by analysis of mRNA expression levels^{21;22;35;36} that indicate increased expression levels of ST6Gal-1. However, these studies did not attempt to identify individual glycans that featured such alterations. As shown in our study, this information is feasible to acquire through the approach of selective amidation of α 2,6-linked sialic acids and lactonization of α 2,3-linked sialic acids, with a subsequent permethylation. In general, it appears that the smaller glycans possessing fewer

(approximately two or less sialic acid residues) undergo more dramatic alterations in their sialic acid linkages than those with more sialic acids during the late-stage breast cancer (see Table 1).

Increasing the level of α 2,6 sialylation has been attributed to higher-activity levels of ST6Gal-1, while these elevated levels have been proven to influence a number of factors that increase the metastatic ability of the tumor cells including decreased cell-cell interactions, increased adhesion to collagen, and increased invasiveness. Therefore, sialyltransferases have been studied as potential targets for drug development.³⁷⁻³⁹ Treatment of mouse and human colon cancer cell lines with KI-8110, a specific sialyltransferase inhibitor, has resulted in a significant reduction of cell-surface sialylation and has been shown to dramatically reduce the formation of metastases in the liver and lungs.⁴⁰⁻⁴² Thus, the ability to monitor alterations in the ratios of sialylated glycan isomers in patients diagnosed with breast cancer may also give an indication of the success of a drug treatment program.

Many human tissues express ST6Gal-1 with significantly different levels. For example, the liver is known to possess higher levels of ST6Gal-1,^{43;44} while very low levels of this enzyme are found in the colons of healthy patients.^{19;45;46} However, the concentrations of ST6Gal-1 are drastically elevated in colorectal carcinomas, leading to their high metastatic potential.^{19;45;46} Moreover, increased levels of this enzyme have been associated with poor prognoses for the patients diagnosed with colorectal cancer.^{19;35;46} These results mirror the findings of Recchi *et al.* who correlated the elevated levels of ST6Gal-1 to ST3Gal-3,²² an enzyme responsible for attaching α 2,3-linked sialic acids to galactose, with a poor prognosis for breast cancer patients. Similarly, the activity levels of ST6Gal-1 are significantly up-regulated for liver diseases, including hepatocellular carcinoma and cirrhosis.^{20;35} Interestingly, the activity of liver ST6Gal-1 can be up-regulated by inflammatory responses in the body by stimulation with cytokines including IL-6.^{47;48} Therefore, it is also possible for ST6Gal-1 to function as a positive acute-phase protein. In the late-stage cancer, cytokines are also produced, while mimicking the effects of inflammation and breast cancer is known to result in the production of IL-6.⁴⁹ Therefore, it is not known at this time whether the results we observe are due to the changes induced by increased ST6Gal-1 activity in the breast cells themselves, or if these alterations are the result of an acute-phase response by the liver. Presently, our laboratory is conducting a glycoproteomic study to answer this question. However, even if these changes are the result of an acute-phase response and are associated with the proteins synthesized by the liver and secreted into the circulatory system, valuable information can still be obtained. Monitoring the ratios of α 2,3- to α 2,6-linked sialic acids over a time-course could be useful in demonstrating cancer progression or regression. It could thus provide useful information about a patient's response to a treatment program and its overall effectiveness.

Conclusions

Through the use of an amidation/lactonization reaction and a subsequent permethylation, sialic acids were differentially labeled, resulting in a mass shift between the isomers and was observed by MALDI-TOF-MS. Sialic acids with α 2,3 linkages spontaneously formed lactones, that were then cleaved during permethylation, while those that were linked α 2,6 were amidated. The newly-formed amide was then doubly-permethyated, resulting in a mass shift. When this method was applied to blood sera samples derived from ten cancer-free and ten late-stage breast cancer patients, a general trend towards isomers with the higher levels of α 2,6-linked sialic acid was noticed. Many sialylated glycans that did not show diagnostic relevance, when based on the glycomic profiling analysis alone, did indicate the presence of the disease after the analysis of sialic acid linkages. These indicators could lead

to a development of methodologies complementing the more conventional glycomic methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Reaction scheme for amidation/lactonization and a subsequent permethylation for (a) α2,3-linked sialyllactose, demonstrating lactone formation and (b) α2,6-linked sialyllactose, resulting in amidation.

Figure 2. MALDI-TOF-MS spectra demonstrating the selectivity and efficiency of the amidation/lactonization reaction for (a) α 2,3-linked sialyllactose and (b) α 2,6-linked sialyllactose.

Figure 3.

Representative MALDI-TOF-MS spectrum of a 2.5- μ l aliquot of human blood serum after amidation/lactonization and subsequent permethylation. The symbols used in this figure: blue square, N-acetylglucosamine; green circle, mannose; yellow circle, galactose; purple diamond, sialic acid; red triangle, fucose.

Figure 4.

(a) Glycomic analysis of a biantennary-disialylated glycan present in blood serum glycoproteins; (b) representative MALDI-TOF-MS spectra of a cancer-free patient (top) and a breast cancer patient (bottom); and (c) notched-box plots for ten cancer-free patients and ten breast cancer patients. The symbols in this figure are the same as those used in Figure 3. In this figure, sialic acid directed toward the left are linked α2,3 while those directed toward the right are linked α2,6.

Figure 5.

(a) Glycomic analysis of a fucosylated biantennary-disialylated glycan present in blood serum glycoproteins; (b) representative MALDI-TOF-MS spectra of a cancer-free patient (top) and a breast cancer patient (bottom); and (c) notched-box plots for ten cancer-free patients and ten breast cancer patients. The symbols in this figure are the same as those used in Figure 3. In this figure, sialic acid directed toward the left are linked α 2,3 while those directed toward the right are linked α 2,6.

Figure 6.

(a) Glycomic analysis of a triantennary-trisialylated glycan present in blood serum glycoproteins; (b) representative MALDI-TOF-MS spectra of a cancer-free patient (top) and a breast cancer patient (bottom); and (c) notched-box plots for ten cancer-free patients and ten breast cancer patients. The symbols in this figure are the same as those used in Figure 3. In this figure, sialic acid directed toward the left are linked α2,3 while those directed toward the right are linked α2,6.

Table 1

Glycan structures included in this study. Percentages of α 2,3-linked and α 2,6-linked sialic acids for each glycan are included along the results of ANOVA and ROC statistical analyses.

Glycan	<i>m/z</i>	# of α 2,3	# of α 2,6	Disease-free		Breast Cancer		P-Value	AUC
				% Rel Int	RSD	% Rel Int	RSD
2431.6	1	0	42.0	10.8	37.5	7.8	0.0224	0.8
	2444.6	0	1	58.0	8.2	62.5	4.3	0.0269	0.2
2605.8	1	0	50.4	16.8	47.1	6	0.323	0.61
	2618.8	0	1	49.6	8.5	52.9	17.1	0.323	0.61
2792.9	2	0	43.0	17.3	21.7	19.1	1.69 e-4	0.98
	2805.9	1	1	17.8	16.3	18.3	13.2	0.82	0.14
2818.9	0	2	39.2	17.1	60.0	17.7	1.50E-04	0.048
	2881.0	1	0	56.9	8.4	50.3	9.3	4.6 e-3	0.86
2894.0	0	1	43.1	11.1	49.7	9.33	4.6 e-3	0.86
	2967.0	2	0	33.7	10.8	27.7	7.8	0.0236	0.81
2980.0	1	1	18.1	8.2	27.9	4.3	2.56 e-4	0.08
	2993.0	0	2	48.2	16.3	44.4	17.9	0.113	0.72
3055.1	1	0	60.0	11.7	48.7	17.4	4.6 e-3	0.83
	3068.1	0	1	40.0	17.5	51.3	16.5	4.6 e-3	0.17
3242.2	2	0	27.7	24.4	22.2	20	0.035	0.75
	3255.2	1	1	36.1	13	35.4	8.9	0.619	0.14
3268.3	0	2	36.2	10.5	42.4	14.3	0.0157	0.1
	3416.3	2	0	26.2	23.1	23.5	21.2	0.285	0.6
3429.4	1	1	46.9	16.1	55.4	8.7	7.31 e-3	0.16
	3442.5	0	2	26.9	29.6	21.1	20.4	0.0577	0.73
3603.5	3	0	21.5	50	11.2	51.4	0.0161	0.79
	3616.5	2	1	8.6	17.8	6.8	19.7	0.019	0.85
3629.5	1	2	39.4	14.1	40.2	15.1	0.916	0.52
	3642.6	0	3	30.5	19.6	41.8	21.6	3.86 e-3	0.12

Glycan	<i>m/z</i>	# of $\alpha 2,3$	# of $\alpha 2,6$	Disease-free		Breast Cancer		P-Value	AUC
				% Rel Int	RSD	% Rel Int	RSD
3777.6	3	0	19.3	47.5	11.3	43.9	0.0292	0.76
	3790.6	2	1	15.9	12.9	13.1	8.5	3.51 e-3	0.84
	3803.7	1	2	57.9	10.9	69.6	6.9	1.12 e-4	0.04
	3816.7	0	3	6.9	31.9	6.0	21.9	0.302	0.64
4053.7	3	0	25.4	40.8	23.1	36.1	0.672	0.53
	4066.7	2	1	29.4	14.3	23.4	20.7	0.0174	0.83
	4079.9	1	2	35.9	13.8	39.6	9.4	0.0303	0.21
	4092.9	0	3	9.3	24	13.9	16.2	9.75 e-5	0.05
4228.0	3	0	29.9	26.7	20.6	26.4	7.41 e-3	0.85
	4241.0	2	1	31.9	19	35.5	8.1	0.0491	0.24
	4254.0	1	2	27.3	12.4	37.2	12.7	1.97 e-5	0.05
	4267.0	0	3	10.9	23.5	6.7	26.7	0.0279	0.95