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L. Renee Ruhaak[1,*], Sandra L. Taylor[2], Carol Stroble[1,3], Uyen Thao Nguyen[2], Evan A. Parker[1], Ting

Song[1], Carlito B. Lebrilla[1], William N. Rom[4], Harvey Pass[5], Kyoungmi Kim[2], Karen Kelly[3], Suzanne

Miyamoto[3]

[1] Department of Chemistry, University of California Davis. Davis, CA, USA

[2] Division of Biostatistics, Department of Public Health Sciences, University of California Davis.

Davis, CA, USA

[3] Division of Hematology and Oncology, University of California Davis Comprehensive Cancer

Center, Sacramento, CA, USA

[4] Division of Pulmonary and Critical Care Medicine, Department of Medicine, New York University

School of Medicine, New York, USA

[5] Department of Cardiothoracic Surgery, NYU Langone Medical Center, New York, New York, USA

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* To whom correspondence should be addressed:

L. Renee Ruhaak
University of Texas MD Anderson Cancer Center
Department of Translational Molecular Pathology
6767 Bertner Avenue
Unit Number 1013
Room Number S13.8136A
Houston, TX 77030
USA
Phone: 713-745-3469
Email: lrruhaak@gmail.com

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ABBREVIATIONS

FDR   False discovery rate

GENT   Gene expression database of normal and tumor tissues

HCC   Hepatocellular carcinoma

LDCT   Low dose spiral computerized tomography

PGC   Porous graphitized carbon

PLS-LDA   Partial least squares regression with linear discriminant analysis

PNGaseF   Peptide -*N*-Glycosidase F

TOF   Time-of-flight

SPE   Solid phase extraction

ABSTRACT

To decrease the mortality of lung cancer, better screening- and diagnostic tools as well as treatment options are needed. Protein glycosylation is one of the major post-translational modifications that is altered in cancer, but it is not exactly clear which glycan structures are affected. A better understanding of the glycan structures that are differentially regulated in lung tumor tissue is highly desirable and will allow us to gain greater insight into the underlying biological mechanisms of aberrant glycosylation in lung cancer.

Here, we assess differential glycosylation patterns of lung tumor tissue and non-malignant tissue at the level of individual glycan structures using nLC Chip/TOF MS. Using tissue samples from 42 lung adenocarcinoma patients, 29 differentially expressed (FDR <0.05) glycan structures were identified. The levels of several oligomannose type glycans were upregulated in tumor tissue. Furthermore, levels of fully galactosylated glycans, some of which were of the hybrid type and mostly without fucose, were decreased in cancerous tissue, while levels of non- or lowly galactosylated glycans mostly with fucose were increased.

To further assess the regulation of the altered glycosylation, the glycomics data was compared to publicly available gene expression data from lung adenocarcinoma tissue compared to non-malignant lung tissue. The results are consistent with the possibility that the observed N-glycan changes have their origin in differentially expressed glycosyltransferases.

These results will be used as a starting point for the further development of clinical glycan applications in the fields of imaging, drug targeting and biomarkers for lung cancer.

INTRODUCTION

Lung cancer is the leading cause of death for men and women in the United States and worldwide due to the inability to detect early stage disease and ineffective treatments for advanced disease (1). In a large randomized trial, low dose spiral computerized tomography (LDCT) was recently shown to identify early stage lung cancer and as a consequence, reduce lung cancer mortality (2). LDCT is likely to become the first approved screening and early detection test for lung cancer but it is plagued by a high false positive rate (2). There is a need to develop complementary screening and early detection tools based on molecular changes due to tumorigenesis. Given that our knowledge of the molecular biology of smoking induced lung cancer has dramatically increased over the past few years this approach is plausible. To date this effort has been focused on the identification of genomic and/or proteomic signatures in a variety of different specimen types with limited success (3). A broader strategy that incorporates additional cancer characteristics is needed.

Protein glycosylation is one of the major post-translational modifications, and it has been proposed as a new paradigm in biomarker discovery and the search for drug targeting leads (4). Glycans are enzymatically synthesized by glycosyltransferases and, due to the different glycosidic linkages, have a large structural heterogeneity. Glycosylation of the proteins plays key roles in cell-cell and cell-matrix interactions as well as cellular differentiation and proliferation (5-7). It is therefore not surprising that, using numerous lectin-binding studies, glycans have been reported to be differentially expressed in tumor tissue compared to controls (8), and that differential expression of glycosylation related

genes has been described (9). Furthermore, several of the proteins currently used as biomarkers for cancer in blood are highly glycosylated (10) (e.g. CA125 (11), PSA (12), CA19-9), indicating the potential importance of protein glycosylation in cancer, and the potential of protein glycosylation analysis in disease identification and treatment.

Advances in technology, particularly the use of mass spectrometry for glycan profiling and identification (13-16), now allow for a more structure specific strategy (17). Mass spectrometry in itself can only determine a glycan's composition, i.e. the number and type of monosaccharides it consists of, but does not allow identification of the specific glycosidic linkages. The use of a complementary separation technique, such as HPLC is needed to allow structure specific analysis. A recent study addresses the glycosylation of colorectal cancer tissues compared to controls using mass spectrometric techniques (18). Among the differentially expressed glycan compositions observed were decreased levels of structures containing a bisecting GlcNAC, while levels of sulfated and paucimannosidic glycans as well as glycans containing sialylated Lewis epitopes were increased (18).

The glycosylation pattern of the membrane fraction of colorectal cell lines has also been compared to the glycosylation of the membrane fraction of epithelial cells derived from colorectal tumors (19). It was observed that the membrane glycosylation of the cell lines differed from the glycosylation of the tumor cells, indicating that cell lines may not always provide biologically relevant glycosylation patterns. Therefore, further studies towards the characterization of differential glycosylation patterns of tumor tissue compared to non-malignant tissue are highly desirable. By elucidating disparate glycosylation patterns between malignant and non-malignant lung tissue it allows us to gain greater insight into the underlying biological mechanisms of aberrant glycosylation in

lung cancer. We anticipate that these results will then be used as starting point for the further development of clinical glycan applications in the fields of imaging, drug targeting and blood biomarkers.

In this study, we apply a nano HPLC coupled with time-of-flight mass spectrometry (nLC-TOF-MS) based method with a porous graphitized carbon (PGC) stationary phase for the separation of N-glycans released from lung adenocarcinoma tissue samples and non-malignant control tissue from the same individual. The method has been shown to be highly stable (20) with an average inter-day coefficient of variation of 4 %, determined on $\log_{10}$-transformed integrals and we previously determined the differential glycosylation profiles and a candidate biomarker panel for ovarian cancer using this method (21). The N-glycans are analyzed twice: once in unreduced form at the compositional level, and once in reduced form at the level of individual glycan structures, which are identified using our in-house library for structural identification (22). Differential glycan compositions and structures are identified and gene expression data from a published study (23) is used to further address the underlying changes in the N-glycan biosynthetic pathway and to further understand lung adenocarcinoma tumor glycobiology.

EXPERIMENTAL SECTION

Human samples

Forty-two de-identified malignant and adjacent normal lung tissue specimens were obtained from the New York University biorepository. All samples originated from patients who were current or former smokers with a diagnosis of lung adenocarcinoma (Stages I-IIIA). Informed consent was obtained from all 42 patients. The study was conducted in

accordance with institutional ethics committee approval at New York University and the University of California, Davis. Residual tumor and adjacent non-malignant tissue was harvested from the resected lung after routine pathological protocols were completed. Two to three tissue pieces were aliquoted into 1.5 ml Nunc vial, and then immediately placed in liquid nitrogen. After transport in liquid nitrogen, each vial was barcoded, and stored at -80°C until analyzed. All specimens were clinically annotated for age, gender, race, histology, smoking status, pack-years and stage of disease. Fortyone patients had stage I and 1 patient had stage IIIA disease. The average age of the cohort was 70.2 (±11.3) years and consisted of 13 males and 29 females. Six patients were current smokers and 36 patients were former smokers; their average pack-year was 33.2 (±24.7).

Homogenization of lung cancer tissue specimens

All procedures were performed on dry ice to keep the samples cold. Depending on the weight of the tissue specimens, they were cut into either three (if >10.0 mg) or two pieces (if <10.0mg) and all pieces from the same specimen were placed in a single 1.5 ml eppendorf tube. Then samples were washed using 1 ml cold PBS (Sigma-aldrich, St.Louis, MO) by pipette mixing and subsequent aspiration to remove remaining blood. 100 μl of HB buffer (0.25 M sucrose, 20 mM Hepes-KOH and 10 μl 1:10 protease inhibitor cocktail (Roche, Basel, Switzerland) per 10 ml buffer, pH 7.4) was added to each of the samples, which were then homogenized using a Bullet Blender Storm for 3 min at speed 8 using prepacked tubes for hard tissues (Wisbiomed, San Mateo, CA) (24).

N-glycan sample preparation

　　　　To assess the stability of the N-glycan sample preparation procedure, one standard serum sample (Sigma-aldrich, St.Louis, MO) was included after every ten tissue samples and as the first and last sample. The sample preparation procedure for serum is slightly different compared to tissue (see below), but all steps were performed in parallel for both the tissue and standard serum samples to ensure accurate sample preparation of the tissue samples.

Fifty µl of 300 mM NH_4HCO_3 (Sigma-aldrich, St.Louis, MO) with 15 mM Dithiothreitol (DTT, Sigma-aldrich, St.Louis, MO) was added to each of the tissue homogenates, while 25 µl of 200 mM NH_4HCO_3 with 10 mM DTT was added to 11 aliquots of 25 µl of a standard serum sample. Proteins were denatured by heat using four cycles of alternating between boiling water (100 °C) and room temperature (25 °C) water for 15 s each and subsequently glycans were enzymatically released by addition of 2 and 1 µl of PNGaseF (New England Biolabs, Ipswich, MA) to the tissue- and the serum samples, respectively and 16 h. incubation at 37°C. Deglycosylated proteins were precipitated using 600 and 200 µL of ice-cold ethanol for the tissue- and serum samples, respectively. After centrifugation, the supernatant containing the glycans was transferred to a 96-wells plate according to the plate layout in supplementary Figure SF1 and brought to dryness *in vacuo*.

　　　　N-linked glycans released by PNGaseF were purified using graphitized carbon SPE plates with 40 µL bed volume (Glygen, Columbia, MD), essentially as described earlier (20). Wells of the SPE plate were conditioned using 2 x 200 µL of 80% ACN containing 0.1% TFA (EMD chemicals, Gibbstown, NJ), followed by 3 x 200 µL of water. Glycan

samples were reconstituted in 400 μL of water and subsequently loaded onto the wells.

Cartridges were washed using 7 x 200 μL of water and N-glycans were eluted using 2 x

200 μL of 40% ACN containing 0.05% TFA. Samples were dried *in vacuo* and reconstituted

in water prior to analysis. 50 μL water was used for the standard serum samples and

tissue samples <10.0 mg, while 100 μL water was used for tissue samples >10.0 mg.

Glycan reduction

Glycan reduction was essentially performed as described earlier (22). Briefly, 44 μl

of a 2M NaBH$_4$ (Sigma-aldrich, St.Louis, MO) solution in water were added to 44 μl of

reconstituted unreduced glycan sample in a 96 wells plate. The plate was incubated at

65°C for 1 h and the reduced glycans were then immediately purified using PGC SPE as

described earlier. Samples were dried *in vacuo* and reconstituted in 50 μL water prior to

analysis.

nHPLC-chip-TOF-MS analysis

N-glycans were analyzed as described before (20) using an Agilent (Santa Clara, CA)

6200 series nanoHPLC-chip-TOF-MS, consisting of an autosampler, which was maintained

at 8°C, a capillary loading pump, a nanopump, HPLC-chip-MS interface and an Agilent 6210

Time Of Flight mass spectrometer. The chip (Glycan Chip II, Agilent) contained a 9 x 0.075

mm i.d. enrichment column coupled to a 43 x 0.075 mm i.d. analytical column; both packed

with 5 μm porous graphitized carbon (PGC). Upon injection of 1 μL of N-glycan sample, the

sample was loaded onto the enrichment column using 3% ACN containing 0.1% formic

acid (FA, Fluka, St. Louis, MO). Then the analytical column was switched in-line so that the

nano-pump delivered a gradient of 3% ACN with 0.1% FA (solvent A) to 90% ACN with 1.0% FA (solvent B) over 17 minutes at a flow rate of 0.4 μL/min. Positive ions were generated using a capillary voltage of 1850V with a nitrogen gas flow of 4 L/min at 325 °C. Mass spectra were acquired at a frequency of 0.63 spectra per second over a mass window of m/z 400 to m/z 3000. The runorder for the different samples is shown in supplementary Table ST1.

Data analysis

Data analysis was performed using Masshunter® qualitative analysis (version B.03.01, Agilent) and Microsoft® Excel® for Mac 2011 (version 14.1.3, Microsoft), according to previous publications (20, 25). Data was loaded into Masshunter® qualitative analysis, and glycan features were identified and integrated using the Molecular Feature Extractor algorithm. First, signals above a signal to noise threshold of 5.0 were considered. Then, signals were deconvoluted using a tolerance of m/z 0.0025 ± 10 ppm. The resulting deconvoluted masses were subsequently annotated using a retrosynthetic theoretical glycan library which was previously developed (26) and contained 331 possible N-glycan compositions. A 15 ppm mass error was allowed. Glycan compositions, retention times and peak area were exported to csv-format for further evaluation.

Statistical Analysis

Prior to statistical analysis, raw peak areas were total quantity normalized based on the underlying assumption that the total amount of ionized glycans that reach the detector is similar for different samples and glycan profiles for each data set. Glycans detected in

fewer than 70% of samples were discarded from downstream analysis to reduce the bias that could be induced by imputation for missing not at random. Unobserved values for any remaining undetected glycans below the predefined detection limit were imputed as one-half of the glycan-specific minimum of the observed values. Finally, the normalized data were $\log_2$ transformed to reduce the influence of extreme values and to meet homogeneity of variance assumptions. Statistical analyses were conducted in R 3.0.1 language and environment.

We conducted a partial least squares regression with linear discriminant analysis (PLS-LDA) to assess whether glycomic profiles could separate malignant from non-malignant tissue samples. To adjust for covariates, we regressed intensity values on age, gender and smoking history and used the residuals, further scaled to a variance of one, in the PLS-LDA. Leave-one-out cross validation was used to estimate the classification accuracy, sensitivity and specificity of the PLS-LDA using one through 10 latent components.

We conducted a differential analysis to identify specific glycans significantly differentially-regulated between cancer tissue and control samples. Two approaches to the differential analysis were used. First, we used paired sample t-tests for univariate analysis without adjustments for covariates. Second, for multivariate analysis we used a mixed effect model in order to take into account age, gender and smoking history as covariates. For the mixed effect analysis, a random effect was included for each patient to account for correlation of glycan intensities in tissue samples from the same patient. Cancer status of the tissue sample (malignant vs. non-malignant), age, gender and smoking history (pack-years) were included as fixed effects. A chi-square test was used to determine significance

of differences in intensity values between malignant and non-malignant tissue samples. For both analyses, false discovery rates (FDR) were calculated to account for multiple testing.

Gene expression

Landi et al. previously performed gene expression analysis using HG-U133A Affymetrix chips on fresh frozen tissue samples of adenocarcinoma and paired noninvolved lung tissue from current, former and never smokers (23). Preprocessed expression data for 27 glycosyltransferases and glycosidases, were extracted from the GENT database (27). Only matched pairs of current and former smokers were analyzed to mimic the inclusion criteria of the glycomics study. This resulted in a dataset originating from paired malignant and non-malignant samples from 22 patients that were current (12) or former (10) smokers. The gene expression data was analyzed using the same statistical methods for differential analysis as described above.

RESULTS

To assess the differential glycosylation profile of lung adenocarcinoma tissue relative to non-diseased lung tissue, samples were obtained from 42 individuals. Samples from both non-malignant and malignant lung tissue, obtained from the same patient, were analyzed. Standard samples, which were included to assess sample preparation as well as instrument errors showed consistent results, as shown in Supplementary Figure SF2, indicating that the data obtained was of good quality. The glycosylation profiles obtained

from three samples showed very low intensities (seen supplementary Figure SF3) compared to the other samples during the assessment of data quality and were excluded from all further statistical analyses. Because these samples were low in both the reduced and reduced analyses, it is not likely that these low intensities were due to instrument failure, but rather sample preparation difficulties. An overview of the patient characteristics of the 39 remaining individuals is provided in Table 1.

Differential glycan compositions in cancer tissue compared to controls

First, a glycan compositional analysis approach was used to assess whether the glycosylation of proteins is altered in adenocarcinoma tissue as compared to control tissue. In this method, glycans were not reduced, resulting in two signals for each glycan due to the separation of reducing-end anomers. Intensities of all signal peaks from a single composition were added to obtain a single representative measure for that composition prior to statistical analysis. Forty-five glycan compositions were consistently (>70% of samples) observed in the tissue samples. These compositions were used to conduct a PLS-LDA analysis to assess whether the global glycomic profile could separate adenocarcinoma tissue from control. The results are shown in **Figure 1a**. While complete separation between the cancerous tissue and the control tissue is not achieved, a clear distinction is observed indicating changes in glycosylation associated with adenocarcinoma.

Differential analysis was performed at the level of individual glycan compositions to identify which glycan compositions contribute to the segregation between the cancerous tissue and the control tissue. Independent of whether the values were adjusted for the covariates, 14 glycan compositions were identified to be differentially expressed (FDR <

0.05) in cancerous tissue compared to controls (**Table 2**). One additional glycan composition ($Hex_5HexNAc_4NeuAc_2$) was shown to be significant when adjusted for the covariates. Interestingly, the levels of several oligomannose type glycans were upregulated. This is concordant with previous studies in human colorectal tissue (18) and mouse breast cancer tissue (28). Furthermore, levels of fully galactosylated glycans, some which were of the hybrid type and mostly without fucose, were decreased in cancerous tissue while levels of non- or lowly galactosylated glycans mostly with fucose were increased. These differences, which are further illustrated in **Figure 2**, have thus far not been reported in lung cancer or other cancer tissue studies.

Differential glycan composition with chemical reduction of glycans

The preceding results showed that glycan compositions were clearly differential in lung cancer tissue compared to controls; however, these compositions are often comprised of several glycan structures (isomers). To further elaborate the glycan structures that are differentially expressed in lung adenocarcinoma tissue, the glycans were reduced to avoid separation of the reducing end anomers and subsequently re-analyzed using nLC-PGC-chip-TOF-MS. Unexpectedly, more glycan compositions were consistently observed in the reduced samples compared to the unreduced set (73 vs 45 compositions, respectively). The reason for the increase in composition is not yet clear. Unreduced glycans separate according to the anomeric character of the reducing end. Each compound is then separated into two forms. Reduction leaves the compound with a single structure thereby decreasing the total number of peaks. The differences could be issues associated with the peak capacity. Further exploration is needed, but this does not affect the results.

The dataset comprising of reduced glycans was assessed at the level of glycan compositions to evaluate whether similar differential results were obtained as compared to the dataset comprising unreduced glycans. The reduced glycan compositions were used to conduct a PLS-LDA analysis to assess whether the global glycomic profile could separate adenocarcinoma tissue from control. The results are summarized in **Figure 1b**. As with the unreduced analysis, separation between the cancer tissues and the control tissues is observed, indicating the differentiating potential of the reduced glycan compositions in adenocarcinoma.

The differential analysis results with and without adjustment for the covariates are shown in **Table 2.** Again, glycan compositions were observed to be significantly (FDR < 0.05) altered in cancerous tissue compared to controls in higher numbers compared to the unreduced samples (22 compositions vs 15 compositions, respectively). However, the results are essentially the same: The levels of several oligomannose type glycans were upregulated. Decreased levels of some hybrid type glycans as well as fully galactosylated glycans mostly without fucose were observed and levels of the N-glycan core ($Hex_3HexNAc_2$) with and without fucose as well as two fucosylated, lowly galactosylated tetraantennary glycans were increased. These results show the similarities of the unreduced and reduced sets and indicate that the analysis of reduced glycans is feasible, thus allowing for glycan structure specific determination of differential glycosylation in adenocarcinoma.

Differential glycan structures in cancer compared to controls

With the glycans reduced, each glycan structure is represented by one signal. This allows for the annotation of actual structures to each of the signals. Structural identification was performed by comparing retention times and accurate masses to an N-glycan database. Here, we used our in-house build library, which is based on serum N-glycans (22) for the annotation of the tissue chromatograms. A typical chromatogram of a cancerous tissue with annotation of the higher abundance glycan structures is shown in **Figure 3**. Although many of the glycan structures are similar to serum glycans, their relative abundances vary from serum. For example, high mannose type glycans are much more abundant in the tissue samples (both cancerous and control) compared to serum (21, 25, 29).

A total of 115 glycan structures were consistently observed in the tissue samples. A PLS-LDA was performed using these structures. There was a clear, but not complete separation between cancerous and control tissues (**Figure 1c**). The separation is similar to what was obtained using glycan compositions. This is further illustrated by an accuracy of classification of 82.0% using the glycan structures, while a 79.5% accuracy was obtained using the unreduced glycan compositions

A differential analysis was also preformed to identify individual glycan structures that show differential glycosylation patterns associated with adenocarcinoma. The results of the differential analyses are shown in **Table 3**. Of the 115 glycans consistently observed in more than 70% of the samples, 29 glycans were shown to be differentially expressed in cancerous tissue compared to controls regardless of adjusting for the covariates. Again, several oligomannose structures as well as structures with low levels of galactose were upregulated in cancerous tissue, while galactosylated structures of hybrid and complex

type were downregulated. For several glycan compositions multiple structures were shown to be significant. While the structures of the same composition were typically all altered in the same direction, the two glycans with composition $Hex_4HexNAc_3Fuc_1NeuAc_1$ were differentially expressed in opposite directions. Because of the opposite expression, composition $Hex_4HexNAc_3Fuc_1NeuAc_1$ was not significant in the compositional analysis, and the differential expression of these glycan structures would thus be missed in a compositional analysis. These results therefore show the potential of the glycan structure specific differentiation. The specific structures of the two differentially expressed glycans of composition $Hex_4HexNAc_3Fuc_1NeuAc_1$ are not yet known. Therefore, further structure elucidation studies will be necessary to evaluate the full biological effects of the different glycan isomers.

Expression of glycosylation-related genes in adenocarcinoma

Glycans are the product of glycosyltransferases and therefore it is to be expected that differential glycan patterns are reflected in differential glycosyltransferase expression. To further assess this relationship, gene expression profiles from literature were compared to the glycomics analysis. Landi et al. previously performed gene expression analysis using HG-U133A Affymetrix chips on fresh frozen tissue samples of adenocarcinoma and paired noninvolved lung tissue (23). Data from 27 glycosyltransferases were extracted, which contained all enzymes relevant to N-glycan terminal processing that were available. Because Landi et al. presented no statistical analysis of the glycosyltransferases, we performed differential expression analysis. Of the 27 genes examined, 15 genes (MAN1A1, MAN1A2, MAN1C1, MAN2A1, MAN2A2, MGAT1,

MGAT2, MGAT3, MGAT4B, B4GALT2, FUT1, FUT2, FUT3, FUT6 and FUT8) were found

differentially expressed (FDR<0.05) in the differential analyses, with and without

adjustment for age and gender as covariates (**Table 4**).

DISCUSSION

This report describes a detailed analysis of the differential N-glycosylation profile

of early stage lung adenocarcinoma tissue compared to paired non-malignant lung control

tissue. Samples were chosen to evaluate the early indications of glycosylation changes due

to tumorigenesis. Glycosylation profiles were first obtained from the native, unreduced

glycans, but to allow for structural identification, a second analysis was performed upon

reduction. This yielded 29 differentially expressed glycan structures. To further assess the

regulation of the altered glycosylation, the glycomics data was compared to gene

expression data from lung adenocarcinoma tissue compared to non-malignant lung tissue,

which was publicly available (23). While it would have been more ideal to have gene

expression data on the same tissue samples, we did not have more tissue material

available.

By analyzing unreduced glycans we have previously been able to identify

differential glycosylation patterns in the serum of ovarian cancer patients (21, 30), lung

cancer patients (29) and prostate cancer patients (25). However, structural identification

is difficult when N-glycans are not reduced due to the separation of the reducing end

anomers. In this study, we showed that the analysis of reduced N-glycans yields very

similar, though extended results, as more compositions can be observed. This resulted in

more significantly different compositions (**Table 2**). Furthermore, the separation of

reduced N-glycans using a PGC stationary phase in combination with our in-house N-glycan library allowed us to identify for the first time 29 glycan structures that were differentially expressed in lung cancer tissue. Notably, two glycans of composition $Hex_4HexNAc_3Fuc_1NeuAc_1$ were shown to be differentially expressed in the opposite direction (**Table 3**). The level of the overall composition was therefore not significantly altered (**Table 2**). This indicates the value of structure specific analysis for the identification of differentiating glycans. However, when the predictive value of the three datasets (unreduced N-glycan compositions, reduced N-glycan compositions and reduced N-glycan structures) were compared, they each yielded very similar results, indicating that for differential profiling (i.e. biomarker) studies a composition specific analysis is likely sufficient.

The glycomics analysis indicated that high mannose type glycans are consistently increased in lung adenocarcinoma tissue (**Tables 2 and 3**). Potentially this could be caused by decreased levels of MGAT1, which indicate lower transformation of high mannose type glycans to hybrid type glycans (**Table 4, Figure 4**). Increased levels of high-mannose type glycans have previously been associated with breast cancer progression (28). Levels of high mannose type glycans were also enhanced upon TGF-β induced epithelial-mesenchymal transition in mouse mammary gland epithelial cells (31), indicating a possible role in cancer progression. However, in serum samples of ovarian cancer patients, high mannose type glycans were shown to be decreased (21, 32). This is likely a reflection of the largely different protein profile of serum compared to tissue.

The level of fucosylation, especially core fucosylation, was also increased in cancer compared to non-malignant tissue (**Table 2, Figure 2**). Comparison to previously

established gene expression data indicated a possible regulatory role for FUT 8, as increased expression of FUT8, the fucosyltransferase that is known to catalyze the addition of the α1-6 linked fucose to the core GlcNAc (33) was observed (**Figure 4, Table 4**). Increased levels of core fucosylation have previously been observed in sera of prostate cancer patients (34). Furthermore, increased gene expression levels of FUT8 have been observed in several cancers including lung (35), ovarian (36), thyroid (37) and colorectal (38) cancers, and that was associated with poor prognosis in colorectal cancer (38). Recently, a mechanism for the upregulation of FUT8 during the transition from epithelial to mesenchymal type cells was proposed (35), and it is anticipated that FUT8 or core fucosylated cancer specific antigens could be excellent drug targets. Indeed, core fucosylated proteins have been proposed as biomarker for hepatocellular carcinoma (HCC) (39-41); however, a recent study did not find increased levels of core fucosylation in HCC tissue (42).

In this study we found levels of hybrid type glycans to be decreased in lung adenocarcinoma tissue. This finding was supported by the decreased expression of MGAT1 and the increased expression of MGAT2 in adenocarcinoma tissue (**Table 4**), which would theoretically result in decreased levels of hybrid type glycans (**Figure 4**). We previously reported on decreased levels of four hybrid type glycans in sera of ovarian cancer patients (21), but to our knowledge, hybrid type glycans have not typically been identified to be differentially expressed in cancer.

Galactosylation is well known to be affected in serum of individuals with several inflammatory diseases; particularly the galactosylation of immunoglobulin G is typically decreased (43-46). Here, we report decreased levels of galactosylation in the tissue

samples of adenocarcinoma patients. The gene expression of one of the enzymes catalyzing

the addition of a galactose to a GlcNAc residue was significantly increased (B4GALT2, see

Table 4), while the expression of the other gene with that functionality was not altered

(B4GALT1) and previous studies indicate that both transferases were upregulated in

breast cancer tissue (9). The gene expression analysis was performed on different samples

which might explain this discrepancy. Gene expression levels do not always correlate with

protein expression and protein activity levels, which could be a reason for the observed

increase of gene expression and decrease of the final product. Another explanation might

be that residual blood was left in the tissue even after thorough washing. It is well known

that lung tissue is well perfused and residual blood might remain. The decreased levels of

galactosylated glycans found in the tissue samples could be a reflection of the decreased

galactosylation previously reported on IgG (43-46). Furthermore, normal lung tissue is

typically better perfused than tumor lung tissue, which might also contribute to the

observation of decreased levels of the in blood typically high abundant biantennary

galactosylated glycans.

Overall, this study describes, for the first time, actual glycan structures –in addition

to compositions- that are differentially expressed in adenocarcinoma tissue compared to

non-malignant tissue from the same individual. These tissues did not only consist of tumor

cells, but also contained the surrounding stroma and other components of the tumor cell

microenvironment. Glycomics changes may therefore partially reflect glycoproteins from

the tumor microenvironment. Further studies will be necessary to identify proteins to

which the glycans are attached. Such studies could be similar in nature to a recent report

on the identification of the peptide moieties of differentially expressed glycopeptides in

pancreatic tissue (47), or could directly target glycopeptides. This will allow for a greater

understanding of possible roles and functions of different glycosylation patterns in the

development and progression of cancer. Glycan profiling of blood samples from these same

lung cancer patients and in a large cohort of early stage patients with matched controls is

ongoing. Data obtained from this study will be valuable in understanding the glycan

composition in the blood and our quest to develop a biomarker for the detection of early

stage lung cancer.

SUPPORTING INFORMATION

Supporting Information Available:

Table ST1. Run order of the lung tissue samples on the nLC-TOF-MS.

Figure SF1. Plate layout used for the glycan analysis from lung adenocarcinoma tissue

samples.

Figure SF2. Quality control of the nLC-TOF-MS run showing the consistency during the MS

runs.

Figure SF3. Total ion chromatograms of the three low-intensity samples and three typical

samples

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FIGURE LEGENDS

Figure 1. Lung adenocarcinoma tissues can be separated from the non-malignant tissue from the same individual based on their N-glycosylation pattern. Score plots of the PLS-LDA analysis are shown for A) unreduced glycan compositions, B) reduced glycan compositions and C) N-glycan structures. Using Leave-one-out cross validation, accurate classification rates were calculated to be 79.5% for the unreduced glycan compositions using 3 latent components, 82.0% for the reduced glycan compositions using 4 latent components and 82.0 for the N-glycan structures using 3 latent components.

Figure 2. Differential expression of glycan compositions in lung adenocarcinoma tissue compared to controls. Putative structures are shown for the glycans that are present at significantly different (FDR<0.05) levels in the unreduced analysis. Glycans of which the levels are decreased in malignant tissue are shown on the left, while glycans of which the levels are increased in malignant tissue are shown on the right Symbol key: blue square is N-acetylglucosamine, green ball is mannose, yellow ball is galactose, red triangle is fucose and purple diamond is N-acetylneuraminic acid.

Figure 3. PGC-LC-MS chromatogram of reduced glycans from a malignant lung tissue sample. The extracted glycan chromatogram (chromatogram of all glycans summed) is shown in black, while extracted ion chromatograms of individual glycans are shown in color. The chromatograms have been annotated with actual structures, as obtained by comparing the retention times to our in-house built serum N-glycan library (22). For symbol key, see Figure 2.

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Figure 4. Schematic overview of N-glycan processing catalyzed by carbohydrate acting enzymes in the Golgi. Enzymes involved in glycan processing have been included, but sugar nucleotide donors have not been included in this figure. For symbol key see Figure 2.

TABLES

Table 1. Sample characteristics of the sample set used for the glycomics analysis in this study.

Variable	LC patients (n=39)
Total sample size, N	39
Age, mean±SD	69.90 ± 11.50
Female, No. (%)	26 (66.67%)
Current Smoker, No. (%)	6 (15.38%)
Pack/Year, mean (± SD)	33.73 (± 24.81)

Table 2. Glycan compositions that are differentially expressed in adenocarcinoma tissue.

GlycanID	*Unreduced analysis*							*Reduced analysis*						
				Unadjusted for covariates		Adjusted for covariates					Unadjusted for covariates		Adjusted for covaraites	
	Tumor Mean[a]	Normal Mean[a]	Ratio[b]	P-value	FDR	P-value	FDR	Tumor Mean[a]	Normal Mean[a]	Ratio[b]	P-value	FDR	P-value	FDR
$H_3N_2F_0S_0$	401031	243979	1.64	0.001	0.004	<0.001	<0.001	632462	437396	1.45	0.009	0.032	0.004	0.018
$H_3N_2F_1S_0$	143561	60381	2.38	0.002	0.010	0.002	0.008	368115	163315	2.25	0.007	0.032	0.002	0.012
$H_3N_4F_1S_0$	282347	162840	1.73	<0.001	0.003	<0.001	0.001	336161	281400	1.19	0.268	0.391	0.259	0.378
$H_3N_5F_1S_0$	157177	82413	1.91	0.015	0.048	0.013	0.040	283220	155035	1.83	0.002	0.015	0.001	0.012
$H_3N_6F_1S_0$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	25853	16755	1.54	0.008	0.032	0.007	0.025
$H_4N_2F_1S_0$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	23247	8457	2.75	0.001	0.008	<0.001	0.002
$H_4N_3F_0S_0$	69856	101298	0.69	<0.001	0.003	<0.001	0.001	183849	238680	0.77	0.178	0.319	0.170	0.304
$H_4N_4F_0S_1$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	45527	70586	0.64	0.009	0.032	0.008	0.027
$H_4N_5F_1S_1$	44795	18954	2.36	0.001	0.005	0.001	0.003	148386	100637	1.47	0.064	0.152	0.053	0.133
$H_4N_6F_1S_0$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	77475	45157	1.72	0.001	0.008	<0.001	0.005
$H_5N_3F_0S_0$	62297	85280	0.73	0.010	0.036	0.009	0.031	120491	165439	0.73	0.046	0.124	0.042	0.113
$H_5N_4F_0S_0$	277677	405445	0.68	<0.001	0.001	<0.001	<0.001	548738	938311	0.58	<0.001	0.002	<0.001	0.002
$H_5N_4F_0S_1$	588178	809015	0.73	<0.001	0.003	<0.001	0.001	1076923	1515725	0.71	0.008	0.032	0.007	0.025
$H_5N_4F_0S_2$	772633	999662	0.77	0.019	0.056	0.013	0.040	1162964	1586227	0.73	0.069	0.152	0.063	0.137
$H_5N_4F_1S_0$	826507	870090	0.95	0.336	0.409	0.327	0.387	1309307	2004782	0.65	0.001	0.010	0.001	0.008
$H_5N_4F_1S_1$	1349183	1697040	0.80	0.001	0.004	<0.001	0.001	2379749	3133061	0.76	0.063	0.152	0.058	0.137
$H_5N_5F_0S_1$	24276	38241	0.63	0.044	0.122	0.040	0.111	33159	66889	0.50	0.003	0.019	0.003	0.013
$H_5N_5F_1S_2$	37578	53028	0.71	0.176	0.269	0.168	0.252	49067	100225	0.49	0.001	0.008	0.001	0.006
$H_5N_5F_2S_0$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	36795	51803	0.71	0.003	0.019	0.002	0.012
$H_6N_2F_0S_0$	658421	482979	1.36	<0.001	0.003	<0.001	<0.001	1026670	723347	1.42	0.010	0.032	0.005	0.020
$H_6N_3F_0S_0$	89282	120565	0.74	0.007	0.028	0.005	0.017	180604	247451	0.73	0.008	0.032	0.007	0.025
$H_6N_4F_0S_0$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	55478	151490	0.37	<0.001	<0.001	<0.001	<0.001
$H_6N_4F_0S_1$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	14364	44239	0.32	<0.001	0.008	<0.001	0.002
$H_6N_4F_1S_0$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	30500	61178	0.50	0.003	0.019	0.002	0.013
$H_6N_6F_0S_0$	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	28979	50194	0.58	0.004	0.022	0.003	0.013
$H_7N_2F_0S_0$	510228	357363	1.43	0.001	0.004	<0.001	0.001	622269	460219	1.35	0.006	0.027	0.002	0.012
$H_7N_6F_1S_2$	20567	30991	0.66	0.140	0.242	0.132	0.221	25926	48286	0.54	0.002	0.017	0.002	0.012
$H_8N_2F_0S_0$	592137	406776	1.46	0.001	0.004	<0.001	0.001	685608	500068	1.37	0.005	0.026	0.002	0.012
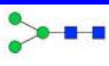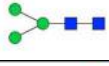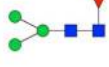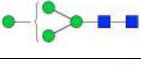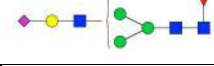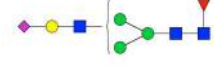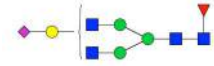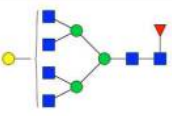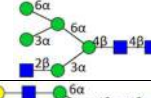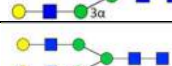
[a] Mean ion intensities are reported for each of the individual glycan compositions.

[b] Ratio between the initial (control) and the final (case) state. Ratio's color-coded in green represent glycan compositions of which levels are decreased in lung adenocarcinoma, while color-coding in red represents glycan compositions of which levels are increased in lung adenocarcinoma.

N.D. indicates that the glycan composition was not determined in the analysis.

Table 3. Individual glycan structures that are differentially expressed in lung adenocarcinoma tissue

Composition	Average RT	Structure[a]	Library entry	Observed mass (da)	theoretical mass (da)	Delta M (ppm)	Tumor Mean[b]	Normal Mean[b]	Ratio[c]	Unadjusted for covariates		Adjusted for covariates	
										P-value	FDR	P-value	FDR
$H_3N_2F_0S_0$	4.86			912.344	912.343	1.1	287152	164841	1.74	0.008	0.034	0.004	0.016
$H_3N_2F_0S_0$	3.64			912.345	912.343	1.2	72518	28613	2.53	<0.001	0.002	<0.001	<0.001
$H_3N_2F_1S_0$	6.19			1058.401	1058.401	-0.7	345819	150017	2.31	0.001	0.008	<0.001	0.003
$H_4N_2F_0S_0$	4.51			1074.393	1074.396	-3.4	54582	39713	1.37	0.004	0.019	0.002	0.012
$H_4N_3F_1S_1$	7.70			1714.632	1714.629	2.0	171879	113106	1.52	0.005	0.026	0.002	0.012
$H_4N_3F_1S_1$	11.07			1714.622	1714.629	-4.0	15486	31287	0.49	0.001	0.006	<0.001	0.003
$H_4N_4F_1S_1$	9.22			1917.710	1917.708	0.8	51559	98025	0.53	0.001	0.008	0.001	0.004
$H_4N_6F_1S_0$	5.86			2032.739	2032.772	-16.2	75633	43708	1.73	0.001	0.006	<0.001	0.003
$H_5N_3F_0S_0$	4.14			1439.529	1439.528	0.5	101534	137078	0.74	0.008	0.034	0.007	0.028
$H_5N_4F_0S_0$	3.15		N5400e	1642.599	1642.608	-5.5	83080	185213	0.45	<0.001	0.001	<0.001	0.001
$H_5N_4F_0S_0$	3.50			1642.596	1642.608	-6.9	44284	99390	0.45	<0.001	0.001	<0.001	0.001

$H_5N_4F_0S_0$	4.93		N5400a	1642.609	1642.608	0.5	330335	485218	0.68	0.001	0.006	0.001	0.004
$H_5N_4F_0S_0$	5.91		N5400c	1642.613	1642.608	3.4	58345	101557	0.57	<0.001	0.001	<0.001	0.001
$H_5N_4F_0S_1$	8.62		N5401d	1933.715	1933.703	5.9	171516	356734	0.48	0.001	0.006	<0.001	0.004
$H_5N_4F_1S_0$	4.28			1788.675	1788.666	5.2	19550	38441	0.51	<0.001	0.002	<0.001	0.001
$H_5N_4F_1S_0$	5.89		N5410a	1788.672	1788.666	3.2	1059476	1623115	0.65	0.001	0.006	0.001	0.004
$H_5N_4F_1S_1$	9.50		N5411e	2079.761	2079.761	-0.2	1359270	1981449	0.69	0.007	0.030	0.006	0.024
$H_5N_4F_2S_0$	5.55			1934.746	1934.724	11.5	24148	12770	1.89	0.002	0.009	0.001	0.007
$H_5N_4F_2S_1$	8.59			2225.809	2225.819	-4.5	22456	57641	0.39	<0.001	0.002	<0.001	<0.001
$H_5N_5F_2S_0$	5.10			2137.816	2137.803	6.2	7940	24773	0.32	<0.001	0.001	<0.001	<0.001
$H_6N_2F_0S_0$	4.21		N6200a	1398.502	1398.502	0.1	928055	655873	1.41	0.001	0.008	<0.001	0.004
$H_6N_3F_0S_0$	4.66		N6300a	1601.582	1601.581	0.3	177960	240773	0.74	0.010	0.039	0.009	0.033
$H_6N_3F_0S_1$	8.45		N6301c	1892.682	1892.677	3.0	46392	92370	0.50	0.002	0.010	0.001	0.008
$H_6N_4F_0S_0$	3.28			1804.659	1804.661	-1.0	26884	67267	0.40	<0.001	0.001	<0.001	0.001
$H_6N_4F_0S_0$	3.81			1804.665	1804.661	2.3	28505	79545	0.36	<0.001	<0.001	<0.001	<0.001

$H_6N_5F_0S_0$	5.37			2007.740	2007.740	0.1	8220	12298	0.67	0.010	0.038	0.008	0.032
$H_6N_5F_1S_0$	7.55			2153.797	2153.798	-0.2	34884	61256	0.57	0.005	0.025	0.004	0.019
$H_7N_2F_0S_0$	4.07		N7200a	1560.551	1560.555	-2.4	544399	403047	1.35	0.012	0.043	0.006	0.025
$H_8N_2F_0S_0$	4.07		N8200a	1722.601	1722.608	-3.7	647154	475956	1.36	0.006	0.027	0.002	0.011

[a] Symbol key: blue square is N-acetylglucosamine, green ball is mannose, yellow ball is galactose, red triangle is fucose , purple

diamond is N-acetylneuraminic acid and open ball is undefined hexose.

[b] Mean ion intensities are reported for each of the individual glycans.

[c] Ratio between the initial (control) and the final (case) state. Ratio's color-coded in green represent glycan compositions of

which levels are decreased in lung adenocarcinoma, while color-coding in red represents glycan compositions of which levels

are increased in lung adenocarcinoma.

Table 4. Differential analysis of gene expression of N-glycan differentiating genes in

paired adenocarcinoma and non-malignant tissue samples.

| GENE | Unadjusted for covariates | | Adjusted for covariates | | Means | | |
	P-Value	FDR	P-Value	FDR	Cancer	Normal	Ratio[a]
MAN1A1	**0.005**	**0.009**	**0.003**	**0.006**	365	527	**0.69**
MAN1A2	**<0.001**	**<0.001**	**<0.001**	**<0.001**	98	68	**1.43**
MAN1B1	0.264	0.264	0.247	0.247	223	197	1.13
MAN1C1	**<0.001**	**<0.001**	**<0.001**	**<0.001**	273	414	**0.66**
MAN2A1	**0.011**	**0.014**	**0.004**	**0.006**	881	610	**1.44**
MAN2A2	**0.011**	**0.014**	**0.005**	**0.006**	195	248	**0.79**
MGAT1	**<0.001**	**0.001**	**<0.001**	**<0.001**	819	1064	**0.77**
MGAT2	**<0.001**	**<0.001**	**<0.001**	**<0.001**	461	320	**1.44**
MGAT3	**<0.001**	**<0.001**	**<0.001**	**<0.001**	57	91	**0.62**
MGAT4A	0.032	0.056	0.028	0.049	118	98	1.21
MGAT4B	**<0.001**	**<0.001**	**<0.001**	**<0.001**	466	334	**1.40**
MGAT4C	0.681	0.715	0.674	0.708	23	22	1.06
MGAT5	0.593	0.655	0.584	0.646	16	18	0.90
B4GALT1	0.145	0.203	0.136	0.190	154	127	1.21
B4GALT2	**<0.001**	**<0.001**	**<0.001**	**<0.001**	308	191	**1.61**
FUT1	**<0.001**	**<0.001**	**<0.001**	**<0.001**	141	223	**0.63**
FUT2	**<0.001**	**<0.001**	**<0.001**	**<0.001**	183	87	**2.12**
FUT3	**<0.001**	**<0.001**	**<0.001**	**<0.001**	253	90	**2.83**
FUT4	0.287	0.344	0.276	0.331	107	117	0.92
FUT5	0.295	0.344	0.284	0.331	7	6	1.20
FUT6	**<0.001**	**<0.001**	**<0.001**	**<0.001**	206	138	**1.49**
FUT7	0.109	0.163	0.101	0.151	20	15	1.28
FUT8	**<0.001**	**<0.001**	**<0.001**	**<0.001**	650	229	**2.84**
FUT9	0.026	0.050	0.023	0.044	26	19	1.37
ST3GAL1	0.158	0.208	0.149	0.195	79	97	0.82
ST3GAL2	0.946	0.946	0.944	0.944	53	54	0.99
ST6GAL1	0.065	0.105	0.059	0.095	384	336	1.14

[a] Ratio between the initial (control) and the final (case) state. Ratio's color-coded in

green represent glycan compositions of which levels are decreased in lung

adenocarcinoma, while color-coding in red represents glycan compositions of which
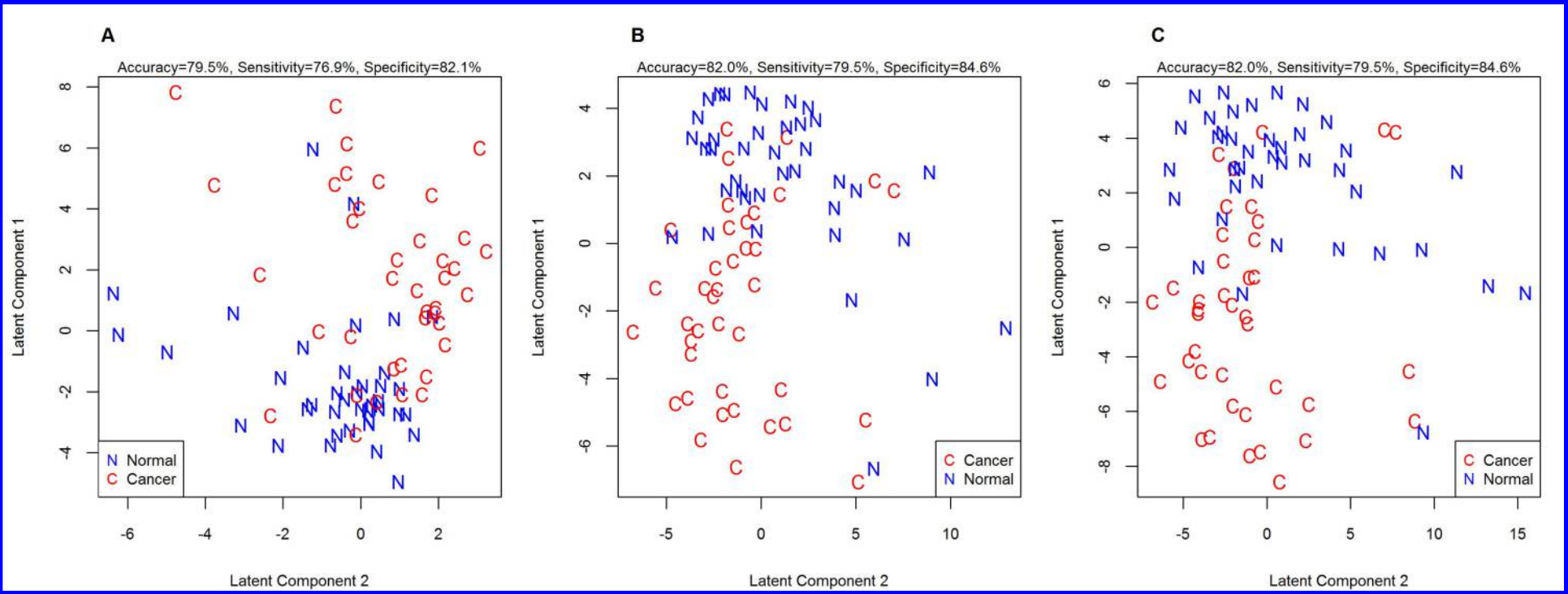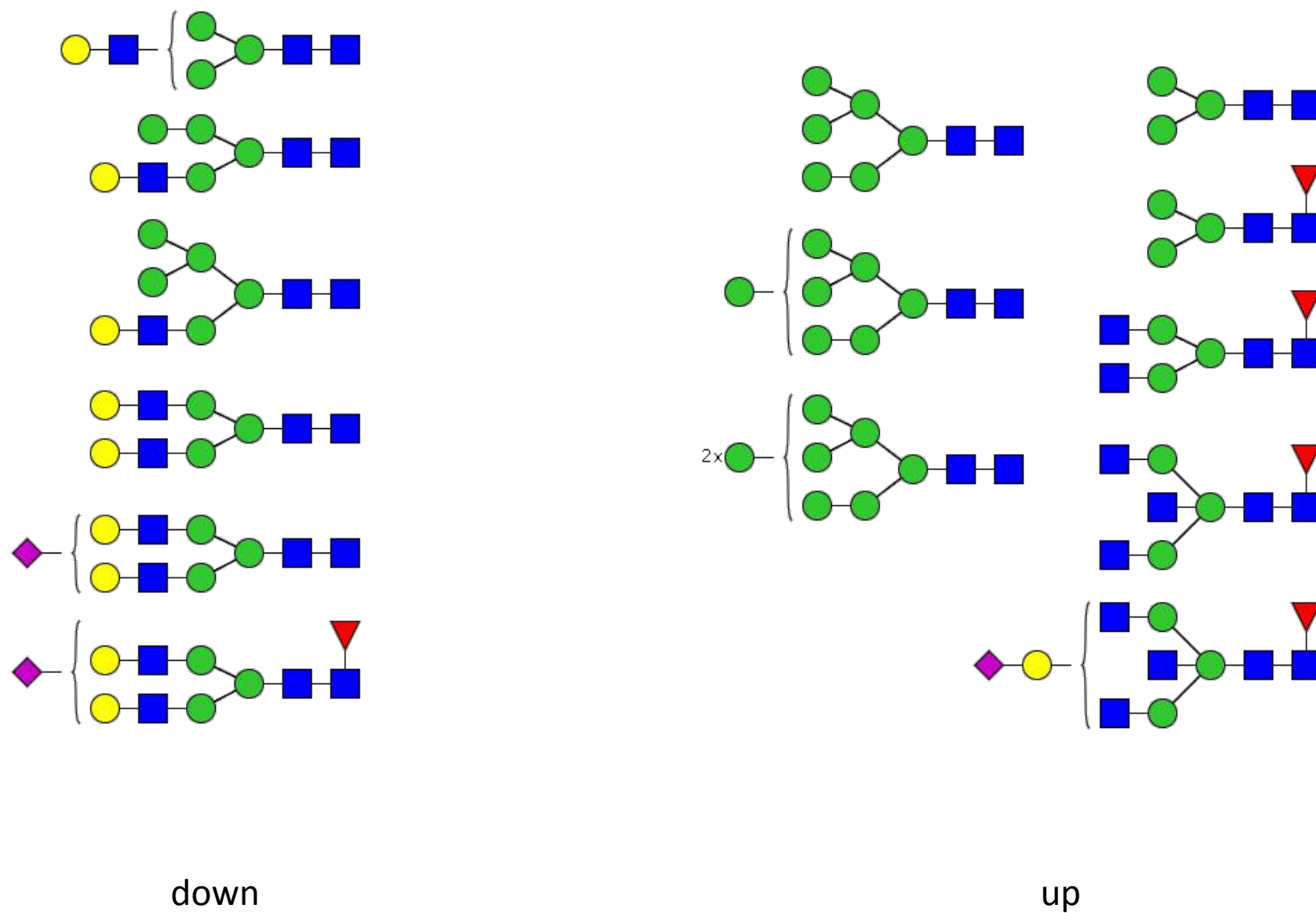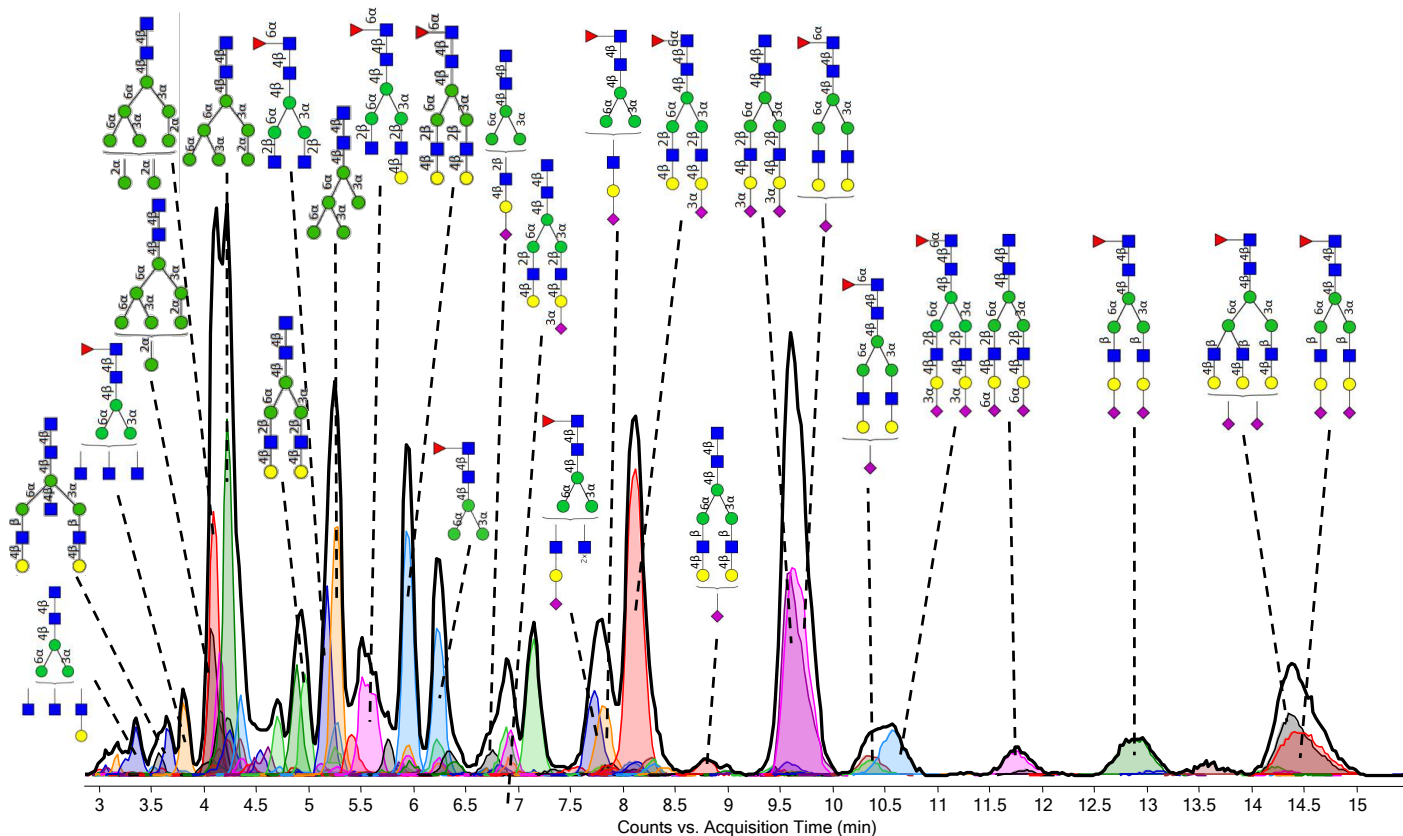
levels are increased in lung adenocarcinoma.

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Figure 1.

down   up

Figure 2.

Figure 3.

Figure 4.

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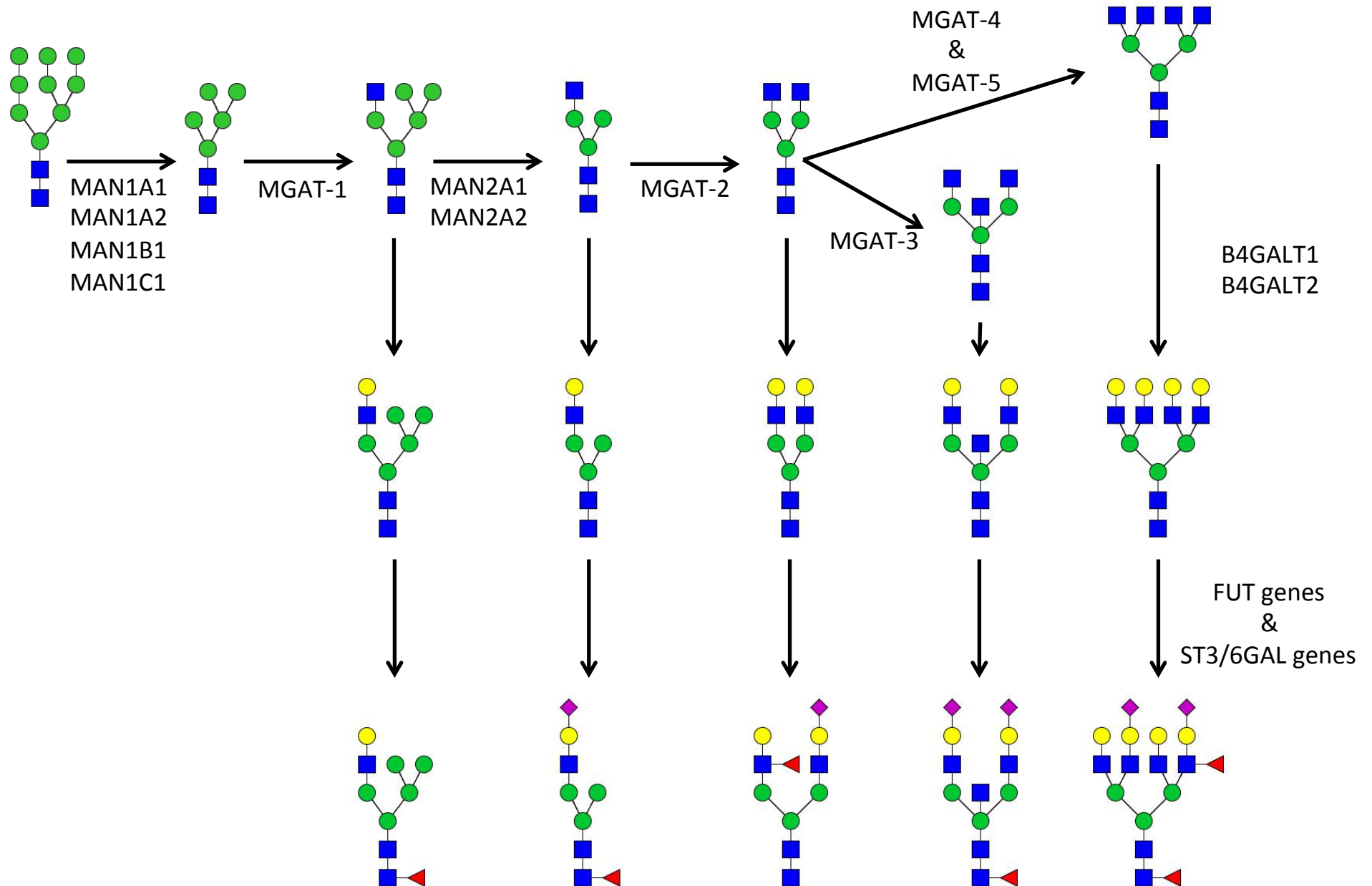
Tumor tissue and adjacent tissue from lung adenocarcinoma patients

42 samples
21 case
21 control

Tissue homogenization
Enzymatic glycan release
PGC SPE purification
Analysis using nLC-TOF-MS

ACS Paragon Plus Environment

Down in tumor tissue

Up in tumor tissue