Glycobiology vol. 18 no. 12 pp. 1105–1118, 2008
doi:10.1093/glycob/cwn095
Advance Access publication on September 25, 2008

A strategy to reveal potential glycan markers from serum glycoproteins associated with breast cancer progression

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Received on June 20, 2008; revised on September 5, 2008; accepted on September 22, 2008

Aberrant glycosylation on glycoproteins that are either presented on the surface or secreted by cancer cells is a potential source of disease biomarkers and provides insights into disease pathogenesis. *N*-Glycans of the total serum glycoproteins from advanced breast cancer patients and healthy individuals were sequenced by HPLC with fluorescence detection coupled with exoglycosidase digestions and mass spectrometry. We observed a significant increase in a trisialylated triantennary glycan containing α1,3-linked fucose which forms part of the sialyl Lewis x epitope. Following digestion of the total glycan pool with a combination of sialidase and β-galactosidase, we segregated and quantified a digestion product, a monogalactosylated triantennary structure containing α1,3-linked fucose. We compared breast cancer patients and controls and detected a 2-fold increase in this glycan marker in patients. In 10 patients monitored longitudinally, we showed a positive correlation between this glycan marker and disease progression and also demonstrated its potential as a better indicator of metastasis compared to the currently used biomarkers, CA 15-3 and carcinoembryonic antigen (CEA). A pilot glycoproteomic study of advanced breast cancer serum highlighted acute-phase proteins α1-acid glycoprotein, α1-antichymotrypsin, and haptoglobin β-chain as contributors to the increase in the glycan marker which, when quantified from each of these proteins, marked the onset of metastasis in advance of the CA 15-3 marker. These preliminary findings suggest that specific glycans and glycoforms of proteins may be candidates for improved markers in the monitoring of breast cancer progression.

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Keywords: breast cancer/*N*-linked glycans/acute-phase proteins/sLe^x/biomarker

Introduction

Breast cancer is amongst the most prevalent cancers in the world and accounts for the highest number of cancer-related deaths among women worldwide. Each year, more than 1.1 million new cases are diagnosed with over 400,000 deaths recorded (http://www.who.int/cancer/bghi.pdf). As in any other malignancy, there is an urgent need for noninvasive markers for diagnostics, evaluating prognosis and monitoring treatment (Chatterjee and Zetter 2005). The most commonly used markers for breast cancer are CA 15-3 and carcinoembryonic antigen (CEA). CA 15-3, however, has low specificity and sensitivity for breast cancer, two important criteria for a biomarker. Therefore, it is often measured together with CEA and only recommended for determining prognosis and monitoring patients (reviewed in Duffy 2006).

Aberrant glycosylation is an established hallmark of cancer and extensive studies have highlighted the role of glycan structures in cancer development (Fukuda 1996; Kannagi et al. 2004). The altered expression of glycan structures on cell surface glycoproteins or in the circulation has also been employed in cancer detection and therapeutic design (Dube and Bertozzi 2005). Tumor markers, such as CA 15-3 and CEA for breast cancer, as well as prostate-specific antigen (PSA-prostate), CA 19-9 (pancreatic) and CA-125 (ovarian), are all glycoproteins with altered glycan profiles in cancer.

The glycosylation of breast cancer has been studied for more than two decades and encompasses various aspects of the glycosylation pathway. Among the most well-studied glycans is sialyl Lewis x (sLex/CD15s), the sialylated form of Lex, also known as CD15. The expression of sLex on leukocytes contributes to their function in the event of an inflammatory response via interaction with E-selectin expressed on endothelial cells. sLex on the surface of malignant cells exploits this mechanism, aiding the extravasation of cancer cells from the blood circulation and their metastasis, with a preference to the liver where E-selectin is highly expressed. The conformational structure of sLex and its binding to the lectin domain of E-selectin via the fucose, galactose, and carboxyl group of the sialic acid is the basis of the development of glycomimetic drugs to inhibit cancer cell metastasis via E-selectin binding (reviewed in Magnani 2004).

Immunohistochemistry of breast cancer tissue suggests sLex expression as an independent prognostic indicator of survival regardless of the size of the primary tumor and lymph node involvement (Nakagoe et al. 2002). Renkonen et al. (1997) compared breast cancer lesions with normal breast tissue in

the same patient and showed that sLex expression on epithelial cells was exclusive to cancerous samples. Concurrently, both P- and E-selectin expression were significantly enhanced on endothelial cells of malignant tissue (Renkonen et al. 1997), consistent with the proposal that sLex binding to selectins aids cancer cell metastasis. It was reported that the high metastatic potential of the RCN H4 colon cancer cells to the liver is due to the expression of cell surface sLex which reduces susceptibility to hepatic sinusoidal lymphocyte-mediated killing (Okuno et al. 2003). The overexpression of sLex on tumor cell surface glycoproteins, however, could have the reverse effect, leading to cytolysis by natural killer (NK) cells via CD94 receptor complex as well as NKG2D, NKG2C, and CD161 (Higai et al. 2006) which recognize sLex (Ohyama et al. 2002). These reports highlight the fact that various levels of sLex expression can lead to different biological consequences.

In this study, we analyzed the serum *N*-linked glycans from advanced breast cancer patients and female controls by a combination of quantitative high-performance liquid chromatography (HPLC) with computer aided data analysis and mass spectrometry (MS) (Royle et al. 2008). We compared the *N*-glycan profiles (117 glycans) from both groups and identified significant changes which highlighted a specific glycan elevated in advanced stage patients. We then performed a retrospective study to evaluate the potential of the glycan as an indicator of metastasis compared with the current clinical marker, CA 15-3. Combining glycan analysis with proteomics, we were able to analyze the glycosylation of a panel of protein spots excised from 2D gels to identify glycoproteins which contributed to the altered glycosylation observed in breast cancer serum. Our preliminary findings suggest their potential as improved biomarkers of metastasis compared with CA 15-3.

Results

The N-glycan pool from advanced breast cancer serum

N-Linked glycans from total serum glycoproteins of 18 advanced breast cancer patients with metastasis (stage 4) and 18 cancer-free controls were analyzed by normal phase (NP)- and weak anion exchange (WAX)-HPLC in combination with sequential digestion using an array of exoglycosidases and MS. We previously identified 117 *N*-glycans in control serum by these methods as described in Royle et al. (2008) and Harvey et al. (2008).

First, we integrated the same 33 glycan peaks in the HPLC profiles obtained in both breast cancer patients ($N = 8$) and controls ($N = 9$) and performed multivariate statistical analysis in order to distinguish the two groups. Based on the percentages of these glycan peaks, we saw a clear separation between the patients and controls (Figure 1A) which was largely due to the levels of core fucosylated biantennary structures in the *N*-glycan pool. This finding suggests that core fucosylation is altered in the glycosylation machinery of glycoproteins in patients with breast cancer, in agreement with the recent report by Kyselova et al. (2008).

Next, each of the *N*-glycan pools obtained from the controls and patients were subjected to WAX-HPLC and the degree of sialylation in every sample was quantified. Interestingly, we found a significant 1.3-fold increase in the tri- ($P = 0.024$) and 1.6-fold increase in tetra- ($P = 0.021$) sialylated fractions of the advanced breast cancer samples compared to controls (Figure 1B).

Consequently, a comparison between breast cancer and control serum protein *N*-glycans showed that the breast cancer *N*-glycans contained increased levels of outer arm fucosylation and that the fucose residue was α1,3-linked to the terminal GlcNAc on the trisialylated triantennary structure (A3FG3S3; GU value of 10.75) (Figure 2A). An α1,3-linked fucose residue attached to GlcNAc at the nonreducing terminus of A3G3S3 constitutes the sLex epitope.

Fractionation of the glycan pool was performed based on total charge (degree of sialylation) again by WAX-HPLC followed by NP-HPLC. This method enables a detailed comparison of structures from each differently charged fraction (with 0–4 sialic acid residues) and, as shown in Figure 2A, highlights that the increase in the α1,3-fucosylated triantennary glycan is in the trisialylated fraction, confirming our primary finding.

The increase in outer arm fucosylation in the patients was also detected by negative ion MS/MS on the desialylated serum *N*-glycan pool. In this analysis, the ratio of outer arm fucose to core fucose on the triantennary glycan $(Hex)_6(HexNAc)_5(dHex)_1$ as detected by the $^{2,4}A_6$ ions (Domon and Costello 1988[22] nomenclature) at *m/z* 1843.6 (core fucose) and 1989.7/1990.6 or 1990.7 (Lewis x structure) was higher in the advanced breast cancer samples than in controls (Figure 2B).

Sequencing and quantification of the glycan marker, A3FG1

Due to the complexity of the HPLC profile and the co-elution of the glycans on the column, the separation of the Lewis x-containing triantennary glycan from other glycans was achieved by digesting with an array of exoglycosidases to segregate and quantify this potential marker as well as to confirm specific linkages. Following digestion with sialidase and β-galactosidase, the α1,3-fucosylated trisialylated triantennary structure (A3FG3S3, GU 10.75) digested to form the α1,3-fucosylated monogalactosylated triantennary structure (A3FG1). This digestion product eluted at GU 7.5 (Figure 3A) as a clear baseline-separated peak allowing accurate quantification. The presence of an outer arm fucose residue hinders the cleavage by the β-galactosidase of the galactose that is linked to the same GlcNAc as the fucose, resulting in the product, A3FG1.

As the linkage of the outer arm fucose and galactose (linked to the same GlcNAc) determines whether the structure is a sialylated Lewis x (α1,3-linked fucose, β1,4-galactose) or Lewis a (α1,4-fucose, β1,3-galactose), it was crucial to distinguish the specific linkages of the glycan marker. Therefore, a combination of both α1,3/4-fucosidase and β1,4-galactosidase digestion was performed on the glycan pool. This array digested the A3FG1 peak completely, confirming the terminal epitope as a sialylated Lewis x (Figure 3A). This was consistent with exoglycosidase digests analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) which showed the disappearance of the A3FG1 ions at *m/z* 1851.8 ($[M+Na]^+$ ion from the reduced glycosylamine – see footnote to Figure 3B – and *m/z* 1970.8 ($[M+Na]^+$ ion from the 2AB derivative) to A3 (*m/z* 1543.6 and 1662.6, respectively, after loss of a galactose and α1,3-fucose) following incubation with α1,3/4-fucosidase (AMF), sialidase, and β1,4-galactosidase (Figure 3B).

Negative ion fragmentation of the monofucosylated triantennary structure was carried out to identify which GlcNAc carries

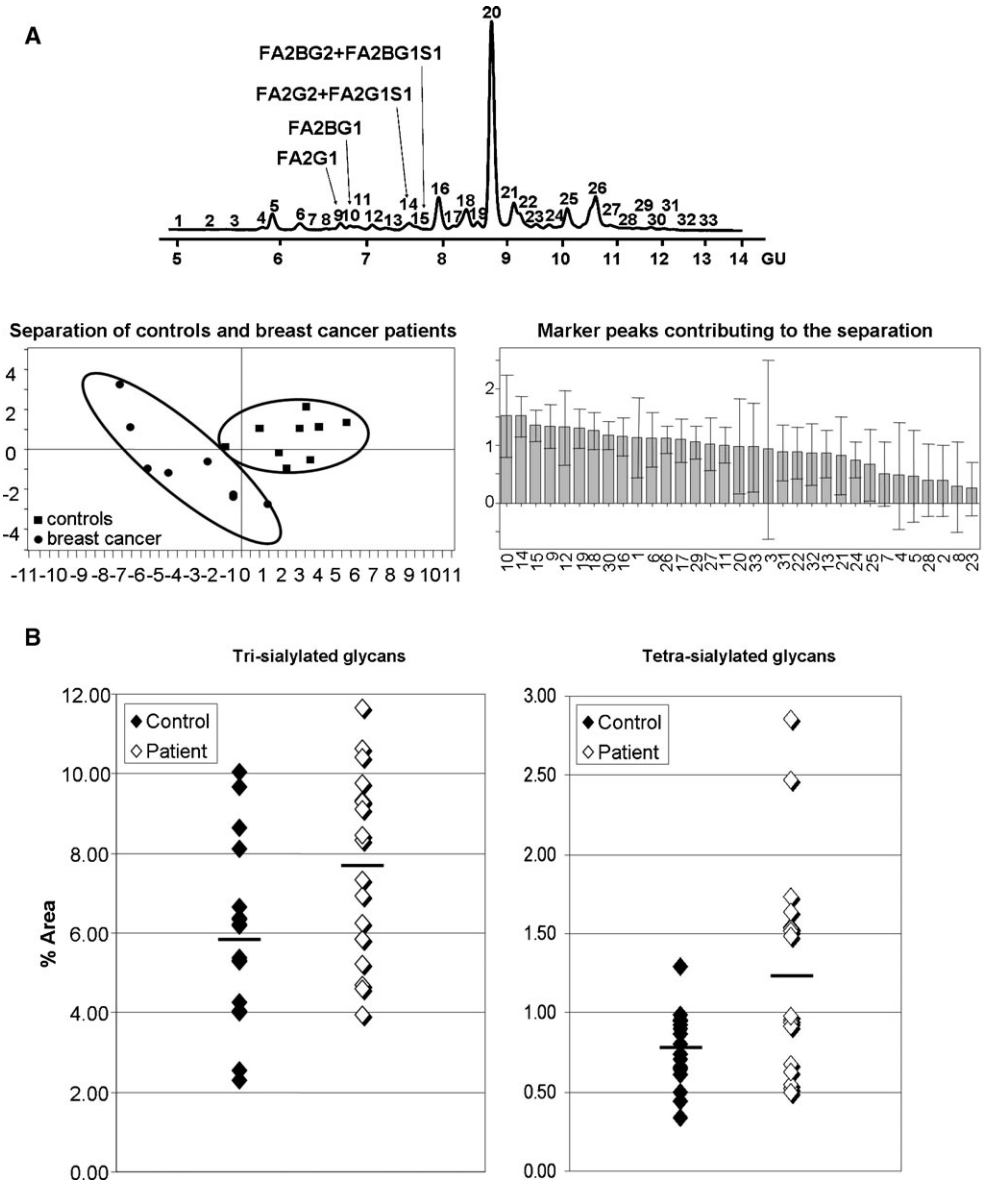

Fig. 1. (**A**) Partial Least Squares-Discriminant Analysis (PLS-DA) plot and importance of variable (individual peaks) plots generated by SIMCA-P+ 12 software (Umetrics, Ascot, UK). The glycan database for whole serum identifying the glycan structures in each peak was determined by Royle et al. (2008). (**B**) Scatter plot of the amount of tri- and tetrasialylated glycans quantified from WAX-HPLC from the same patients. Black bars indicate the average levels for trisialylated glycans in control (5.86 ± 2.21) and advanced breast cancer (7.65 ± 2.33) groups and for the tetrasialylated glycans in control (0.77 ± 0.23) and advanced breast cancer (1.24 ± 0.66) groups.

the outer arm fucose (Figure 3C). The spectrum contained a major E-type ion (Harvey 2005) at *m/z* 977 ($Gal_2Glc_2Fuc_1+101$) indicating fucose substitution in the 3-antenna. The spectrum also contained B_2 cleavage ions (Domon and Costello nomenclature (Domon and Costello 1988)) at *m/z* 364 ($Gal_1GlcNAc_1$) and *m/z* 510 ($Gal_1GlcNAc_1Fuc_1$) and a C_2 cleavage ion at *m/z* 382, consistent with fucose substitution on an antenna. The substitution of the fucose on the GlcNAc residue was confirmed by the absence of a C_1 (Gal-Fuc) ion expected at *m/z* 325 (179 + 146). The spectrum did not indicate which branch of the 3-antenna contained the fucose residue but substitution on the GlcNAc of the 4-linked branch is most probable, based on studies with α1-acid glycoprotein (Fournet et al. 1978).

The branch of the 3-antenna to which the fucose was attached was determined by the method described by Kolarich et al. (2006). The compound present in the peak at GU 7.5 (A3FG1) that was obtained by incubation with sialidase and bovine testis β-galactosidase (BTG) was isolated. The glycan in this fraction was further incubated sequentially with α-fucosidase (AMF), β-hexosaminidase (JBH), and *Streptococcus pneumoniae* β-galactosidase (SPG) to give a compound consisting of the trimannosyl-chitobiose core with one attached GlcNAc residue on the 3-antenna as shown in Figure 3A. This compound had a GU value of 4.95 which was compared to the glycan of the same composition derived from exoglycosidase digestions of the characterized IgG *N*-glycans in which the GlcNAc is

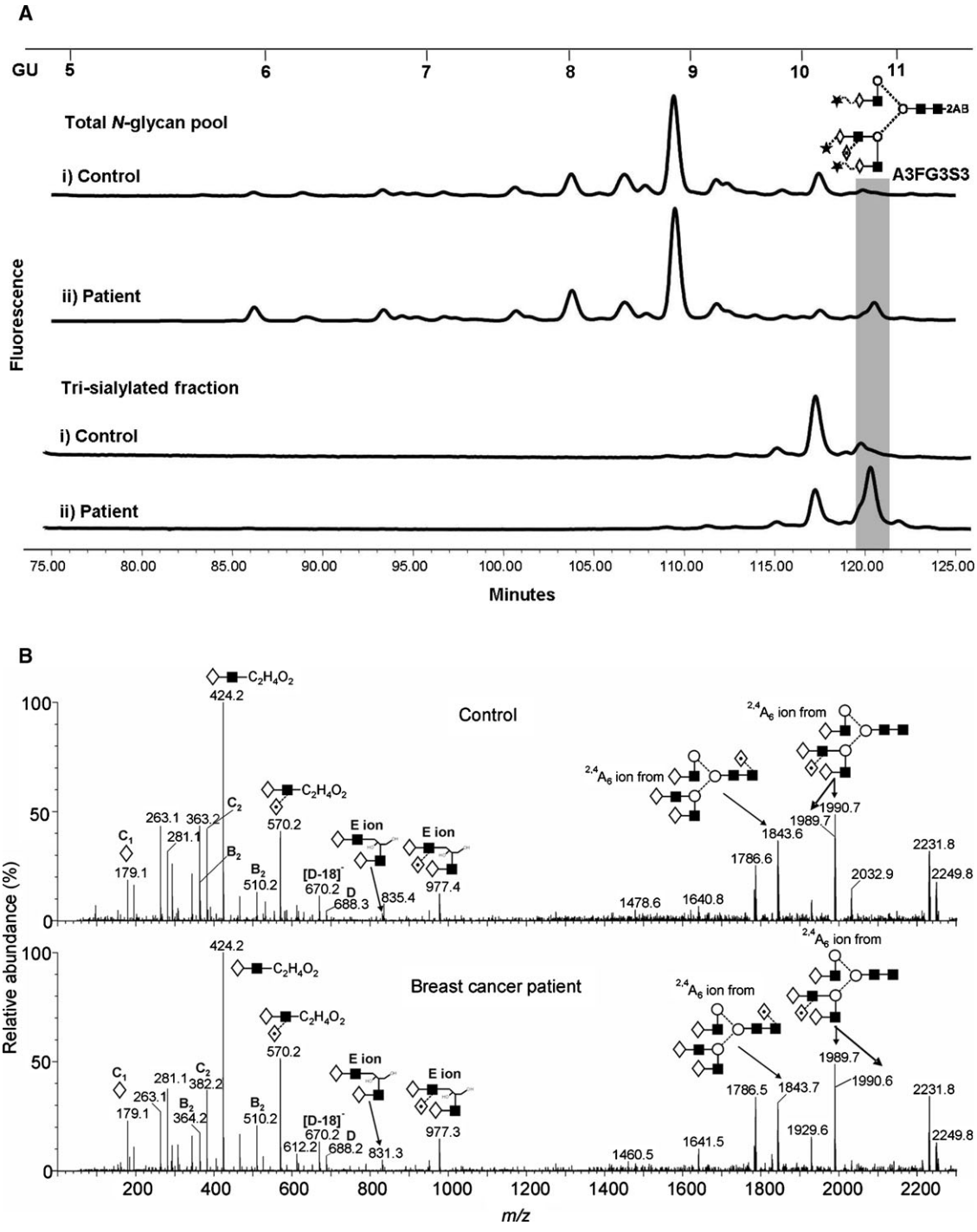

Fig. 2. Normal phase HPLC profiles of *N*-glycans released from the total serum protein from a healthy control and an advanced breast cancer patient. The *N*-glycan pool consists of more than 117 structures, all of which were identified by combining NP-HPLC with exoglycosidase digestions, WAX-HPLC, and mass spectrometry as described in Royle et al. (2008). Glucose units (GU) were obtained by comparing the glycan profiles to a standard dextran ladder. (**A**) Upper panel: the undigested *N*-glycan pool showing the increase in the structure A3FG3S3 at GU 10.75 in breast cancer and lower panel: NP-HPLC profiles of the trisialylated fractions of the *N*-glycan pool from control and patient serum separated by WAX-HPLC amplifying the increase in A3FG3S1 in patient compared to control. Structure abbreviations: all *N*-glycans have 2 core GlcNAc residues; F at the start of the abbreviation indicates a core fucose α1,6-linked to the inner GlcNAc; Man(x), number (x) of mannose on core GlcNAcs; A(x), number (x) of antenna (GlcNAc) on the trimannosyl core; B, bisecting GlcNAc linked β1,4- to the β1,4-mannose; F(x), number (x) of fucose linked α1,3- to an antenna GlcNAc, G(x), number (x) of galactose residues on the antennae; S(x), number of sialic acids on the antennae. All structures were confirmed by exoglycosidase sequencing and also by both MALDI and ESI-MS/MS; all experimental masses were within 0.2 Da of the calculated masses. Symbolic representation of glycans is as follows: GlcNAc, filled square; mannose, open circle; galactose, open diamond; fucose, diamond with a dot inside; beta linkage, solid line; alpha linkage, dotted line; 1-4 linkage, horizontal line; 1-3 linkage, (/); 1-2 linkage, vertical line; and 1-6 linkage, (\). (**B**) The ESI MS/MS spectra of the desialylated fucosylated triantennary *N*-glycans from a control and an advanced breast cancer patient. Abundant ions at *m/z* 265.2 and 553.3 from a contaminant have been removed from the spectrum in the top panel for clarity. Note that some of the higher mass ions are annotated both at the $^{12}C_1$ and $^{13}C_1$ isotopic ions. Details of the fragmentation nomenclature can be found in Figure 3C.

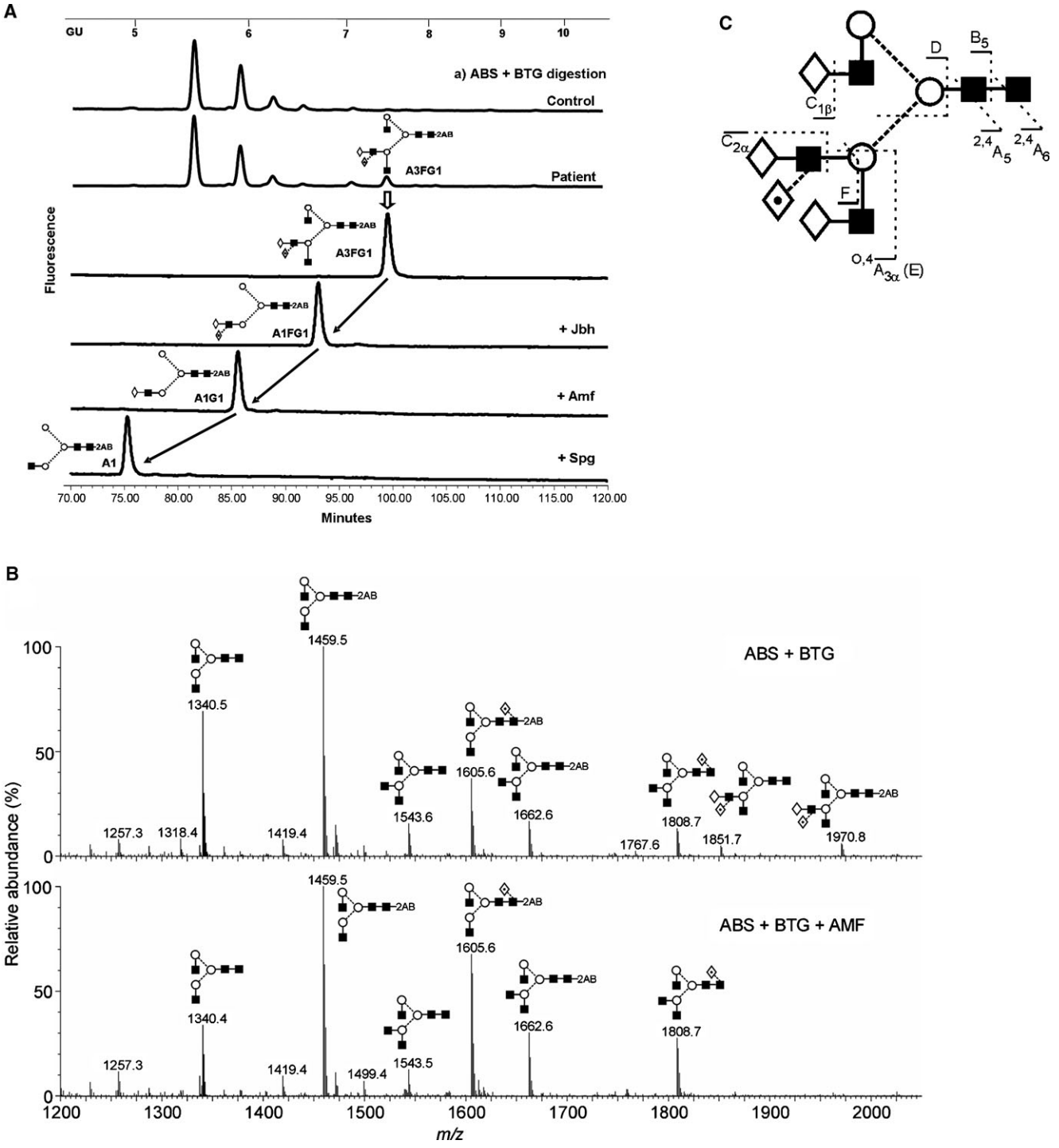

Fig. 3. Identification and quantification of the glycan marker, A3FG1. (**A**) Total *N*-glycan profiles following sialidase and β-galactosidase digestions for quantification of A3FG1 at GU 7.5, the digested product of A3FG3S3. The compound producing the A3FG1 peak was collected and further digested sequentially with AMF, JBH, and SPG to determine its specific linkages based on the resulting GU. ABS, *Arthrobacter ureafaciens* sialidase (removes all sialic acids); BTG, bovine testis β-galactosidase (removes β1-3 and β1-4 galactose); AMF, almond meal fucosidase (removes α1-3,4-linked fucose); JBH, jack bean hexosaminidase (removes GlcNAc); SPG, *Streptococcus* pneumoniae β-galactosidase (removes only β1,4-linked galactose). (**B**) MALDI-TOF MS of the *N*-glycan pool (2AB derivatives) of a patient following ABS and BTG digest (top panel) and further with the addition of AMF (lower panel). The spectra have been processed with the MaxEnt 2 algorithm feature of MassLynx in order to remove base-line noise. The 2AB derivatives were prepared before complete hydrolysis of the glycosylamine, resulting from the PNGase F release, had occurred. Consequently, the glycosylamine was not derivatized but was reduced by the sodium cyanoborohydride to give a product one mass unit higher than the native glycan. Thus, the ions at *m/z* 1851 and 1970 in the top spectrum are from the reduced glycosylamine and 2AB derivative of the A3FG1 glycan, respectively. In the presence of AMF (lower spectrum), both compounds digested completely. (**C**) Negative-ion fragmentation of the monofucosylated triantennary structure.

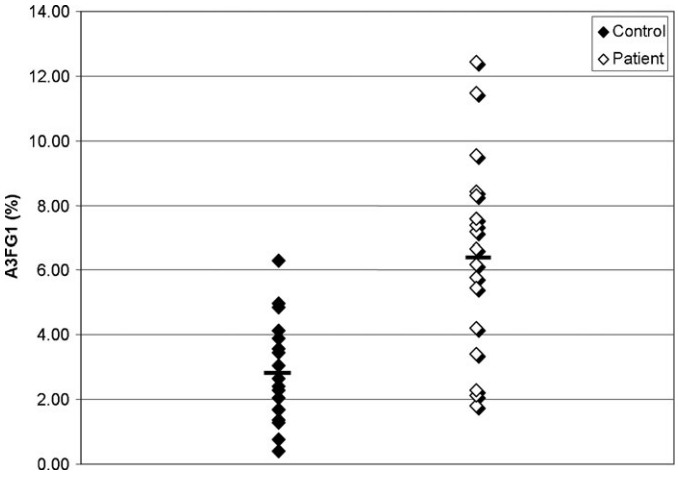

Fig. 4. Scatter plot of the glycan marker. A3FG1 quantified from the *N*-glycan pools of healthy female controls ($N = 18$) and advanced breast cancer patients ($N = 18$). Black bars indicate the average levels for each group: control (2.82 ± 1.59) and advanced breast cancer (6.55 ± 3.02).

α1,2-linked to the 3-antenna. Since this compound had a GU value of 5.1 and was different from that of the glycan derived from breast cancer serum, the α1,3-linked fucose was confirmed to be on the GlcNAc that was β1,4-linked to the trimannosyl core (Figure 3A).

A3FG1 as a biomarker for advanced breast cancer and disease progression

We identified the A3FG1 glycan by NP-HPLC following exoglycosidase digestion of the 2AB labeled total *N*-glycan pool (117 glycans, (Royle et al. 2008)) as shown in Figure 4. The relative percentage area of this peak was measured for all of the advanced breast cancer patients ($N = 18$) and individual female controls ($N = 18$) following digestion with sialidase and β-galactosidase. There was a marked increase of more than 2-fold for the advanced breast cancer (range 3.53–9.57) compared to control (1.23–4.41), significantly separating patients from controls ($P = 0.001$). This control value is somewhat higher than the level measured from the pooled control which was a pool from over 30 mixed-sex healthy individuals (2.2%). To our knowledge, all the 18 individual female controls are free of breast cancer and healthy.

To evaluate the potential of A3FG1 as an indicator of breast cancer progression, a longitudinal case study was performed on 10 individual patients from whom sera were collected at two time points during the malignancy: the earlier sample was taken when breast cancer was first diagnosed and the later after metastases were detected (Table I). A3FG1 as well as the CA 15-3 levels of these samples were plotted separately against the duration of breast cancer (Figure 5A and B). We observed a significant difference in the trends of both A3FG1 and CA 15-3 in all 10 patients. Interestingly, we found that A3FG1 increased in all the second samples, clearly consistent with breast cancer progression and/or metastasis. This increase was in contrast to the CA 15-3, which only showed increased levels in 4 out of 10 patients (the others showed no significant increase and two cases presented reduced levels). The level of this glycan marker was also compared against CEA (Table I) where out of six patients for whom we had measurements, all had insignificant levels that did not indicate disease progression. This preliminary result suggests that compared to the commonly measured CA 15-3 and CEA, the glycan marker A3FG1, quantified from whole serum of breast cancer patients, could be more reliable for detecting disease progression and metastasis.

Glycoproteomics to identify proteins carrying sLex

Total serum proteins from advanced breast cancer and controls were subjected to 2D electrophoresis ($pI = 3$–10) followed by Western blotting using KM93, an antibody against the sLex epitope. We identified three glycoprotein spots in the patients' blot which were not observed in the control (Figure 6A). These spots were excised and subjected to *N*-glycan release for glycan sequencing, followed by digestion with trypsin for protein

Table I. Measured serum markers and staging of breast cancer serum

Patient	Staging	Months of disease	Metastasis	A3FG1 (%)	CA 15-3 (U/mL)	CEA (U)
Patient 1	3	0	–	4.16	317	3
	4	39	Bone, pleura, liver	5.45	67	1
Patient 2	3	0	–	2.93	15	1
	4	14	Liver, brain	4.20	18	1
Patient 3	ND	0	–	3.57	163	ND
	4	40	Liver	7.38	55	67
Patient 4	2	0	–	3.86	103	8
	4	120	Bone	7.58	92	8
Patient 5	ND	0	–	5.54	116	6
	4	78	Pleura, skin	7.58	122	5
Patient 6	3	0	–	2.13	78	1
	4	24	Bone	2.28	78	1
Patient 7	ND	0	–	5.51	30	2
	4	60	Bone	8.31	337	ND
Patient 8	3	0	–	5.64	26	3
	4	50	Skin, bladder	6.18	25	ND
Patient 9	3	0	–	7.34	45	2
	4	23	Skin, bone	9.56	51	5
Patient 10	3	0	–	5.31	82	ND
	4	61	Pleura, liver	6.34	288	8

ND: not determined.

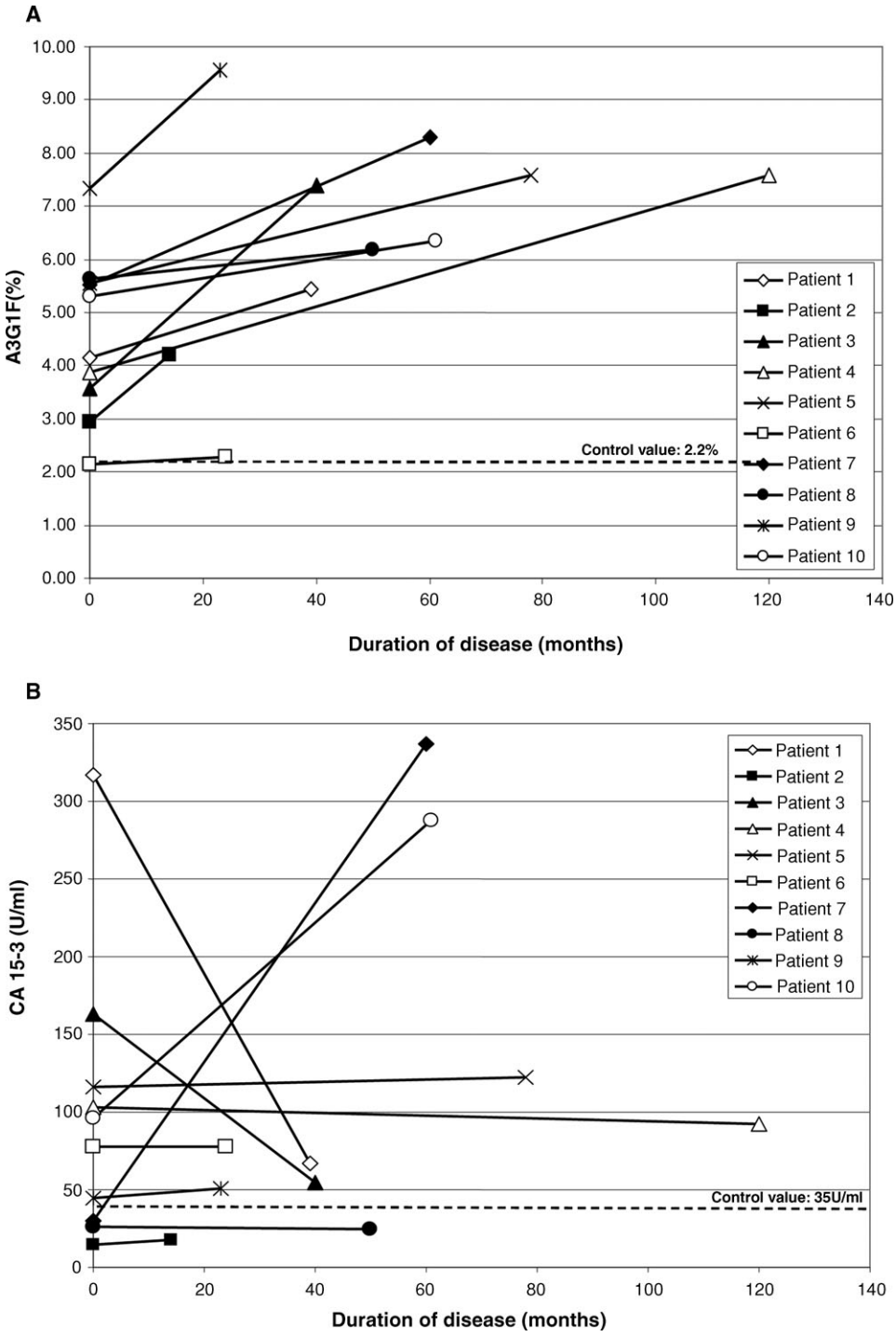

Fig. 5. Longitudinal study correlating the glycan marker A3FG1 and CA 15-3 from whole serum of breast cancer patients ($N = 10$). Two samples were obtained from each patient for evaluation: an early stage sample following diagnosis and an advanced stage sample after detection of metastasis. (**A**) A3FG1 levels quantified by exoglycosidase digestion and NP-HPLC plotted against duration of disease progression. (**B**) CA 15-3 measured by the CA 15-3 automated assay (Bayer Centaur), plotted also against the duration of breast cancer.

identification by LC-MS/MS. All three spots contained the α1,3-fucosylated trisialylated triantennary structure containing sLex epitope (A3FG3S3) (Figure 6A) and the proteins identified are as listed in Table II.

The first spot (i) contained α1-antichymotrypsin (ACT) along with other glycoproteins (kininogen, vitronectin, and corticosteroid-binding globulin) with ACT having the highest score and sequence coverage. In the second spot (ii), two isoforms of α1-acid glycoprotein (AGP), AGP1 and AGP2, were identified together with ACT. In the third spot(iii), haptoglobin β-chain was found together with complements C3 and C4. Although complement C3 scored higher compared to haptoglobin, the sequence coverage for haptoglobin was the higher of the two and we found no evidence of C3 glycosylation (oligomannose

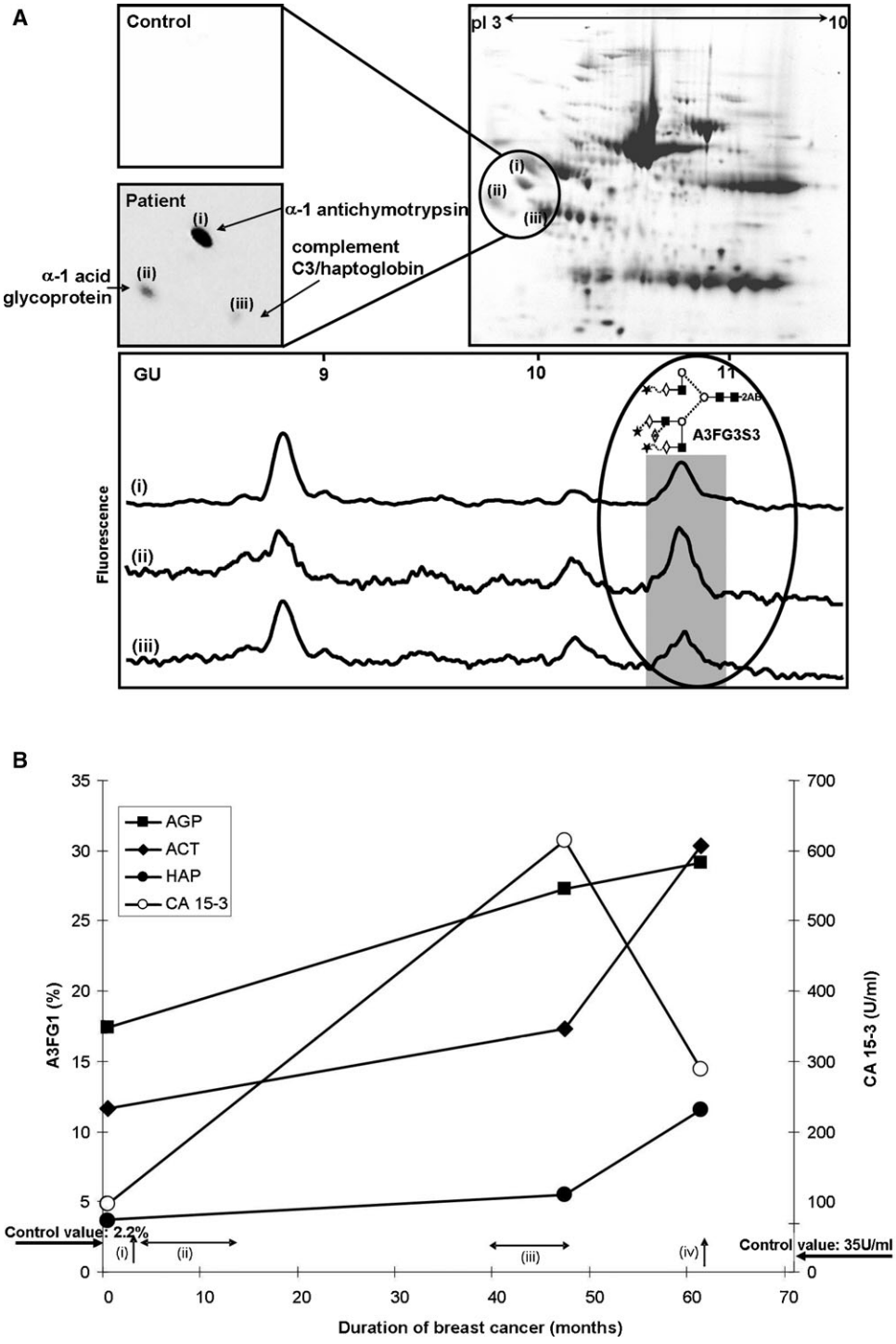

Fig. 6. Glycoproteomics approach to find candidate proteins carrying the serum glycan marker. (**A**) Two-dimensional polyacrylamide gel electrophoresis (PAGE) of breast cancer serum (80 μg) stained with the fluorescent dye, OGT 1238 (Oxford Glycosciences, Abingdon, UK). Duplicate gels of breast cancer and control serum were immunoblotted against sLex using the KM93 antibody, highlighting target proteins and showing structure A3FG3S3 in their undigested *N*-glycan pool (spots i–iii). (**B**) Quantified levels of A3FG1 from the *N*-glycan pools of α1-acid glycoprotein, α1-antichymotrypsin, and haptoglobin from the serum samples of a breast cancer patient, plotted against CA 15-3. Patient timeline: (i) following radiotherapy, mastectomy stage 3 grade 2-invasive breast cancer (at least 100 mm) with tumor in 16 of 20 lymph nodes; (ii) 6 months of adjuvant chemotherapy followed by Tamoxifen and a further combination of Zoladex and Tamoxifen; (iii) pain and rising tumor markers with no evidence of progression in bone scans, ultrasound, and X-rays; and (iv) metastases detected in liver and the right pleura.

Table II. sLex-bearing proteins identified in advanced breast cancer serum

Gel spot	Protein name	Swiss-prot entry	Protein score	Sequence coverage (%)	Numbers of peptides matched
i	Alpha-1-antichymotrypsin precursor	AACT_HUMAN	1010.23	51.84	20
	Kininogen precursor	KNG_HUMAN	418.81	14.90	7
	Corticosteroid-binding globulin precursor	CBG_HUMAN	46.03	6.17	1
ii	Alpha-1-acid glycoprotein 2 precursor	A1AH_HUMAN	162.61	26.36	3
	Alpha-1-acid glycoprotein 1 precursor	A1AG_HUMAN	148.75	25.37	4
	Alpha-1-antichymotrypsin precursor	AACT_HUMAN	76.69	18.43	3
iii	Complement C3 precursor	CO3_HUMAN	575.72	11.00	15
	Haptoglobin precursor	HPT_HUMAN	440.78	28.81	11
	Complement C4 precursor	CO4_HUMAN	45.05	1.66	1

structures) or C4 *N*-glycans (biantennary structures) (Crispin et al. 2004) in our profiles. The A3FG3S3 structure was also present on the same glycoproteins from ovarian cancer patient sera (Saldova et al. 2007) indicating that these markers are not specific to breast cancer.

sLex glycoforms individual proteins as indicators of breast cancer progression

Once we had established glycoproteins (α1-antichymotrypsin, haptoglobin β-chain, and α1-acid glycoprotein) that contributed to the increase in sLex levels in serum using anti-sLex antibody, we quantified and examined A3FG1 levels (digestion product predominantly from A3FG3S3) from each of these proteins to establish whether or not the levels correlated with breast cancer progression. To determine this, we performed a case study on a single breast cancer patient and separated the serum proteins (80 μg, ∼1.5 μL) of three samples from this patient as shown in Figure 6B. We quantified the A3FG1 from NP-HPLC profiles of *N*-glycans released from α1-acid glycoprotein, α1-antichymotrypsin, and the most acidic spot of haptoglobin β-chain excised from each 2D gel. The A3FG1 levels measured from *N*-glycan pools of these proteins were then plotted alongside CA 15-3, quantified from each sample (Figure 6B).

The amounts of both A3FG1 and CA 15-3 from the specific proteins showed marked increases between the samples from early diagnosis and the second samples taken following treatment. However, in the third sample which was collected following the diagnosis of metastasis, we observed that the CA 15-3 level, although still exceeding the normal value (35 U/mL), showed a marked drop in the last point where metastasis was detected compared to the previous time point (Figure 6B), which might falsely indicate improvement. From the same sample, interestingly, the levels of A3FG1 in these samples increased significantly, more accurately reflecting disease progression. This preliminary result suggests that the evaluation of these A3FG1-containing protein glycoforms could also be better indicators of metastasis than CA 15-3 and could serve as an alternative method for early detection of metastasis and for monitoring breast cancer progression.

Discussion

Altered N-glycosylation of glycoproteins in breast cancer serum

Alterations in *N*-linked glycosylation in cancer as well as other diseases have gained a lot of research interest and have shown potential both as a source of disease markers and targets for immunotherapy of tumors (Kobata and Amano 2005). Using our robust and highly sensitive technology, we analyzed the *N*-glycans of total serum proteins from breast cancer in search for aberrant structures that could distinguish between breast cancer patients and controls.

In general, we observed changes in fucosylation which contributed to the separation of the patient group from the controls (Figure 1A). Using WAX-HPLC analysis, we measured a significant increase in the degree of sialylation on the tri- and tetraantennary structures in the advanced breast cancer patients (Figure 1B). We also identified increased levels of trisialylated triantennary structures with α1,3-fucose, which forms part of the sLex epitope, in patients compared to controls (Figure 2A). Synthesis of sLex is known to require sialylation to precede fucosylation of the internal GlcNAc residues by ST3Gal-IV and VI (Holmes et al. 1986; Grabenhorst et al. 1998). The increase in outer arm fucosylation suggests that there is increased activity of sialyltransferases (ST) in breast cancer, as reported previously by measurements of the respective levels in patient serum and tissue, both of which correlated with disease progression (Bosmann and Hall 1974); Stewart et al. 1982). The main fucosyltransferase involved in the synthesis of sLex is FucT VI, whose gene expression correlates with sLex expression on the surface of breast cancer cells (Matsuura et al. 1998).

The gene expression patterns and protein levels of *N*-acetylglucosaminetransferase, GnT-V, ST3Gal, and α1,3-FucT also correlate inversely with nm23-H1, a tumor suppressor gene whose expression results in reduced sLex biosynthesis, lowering the metastatic potential of the breast cancer cells (Duan et al. 2005) and influencing disease-free survival rates of patients (Yamaguchi et al. 1998).

Very recently, a similar study on *N*-glycans of breast cancer serum glycoproteins was published. Using an MS approach coupled with statistical analysis, Kyselova et al. (2008) established a series of glycans that were common between the human patients and breast cancer cell lines. Among those glycans were subsets which increased with disease progression (stages I–IV) and distinguished between invasive and noninvasive cell lines. Interestingly, these consist of mono- and difucosylated sialylated tri- and tetraantennary structures. This technique, however, cannot distinguish linkage of the glycans, perhaps explaining why only 50 glycans were identified whereas our HPLC/MS technology which provides monosaccharide sequence and linkage information identified 117 *N*-glycans from human serum (Royle et al. 2008). Nevertheless, in agreement with our findings, this study concluded that fucosylation was the most significant change observed in breast cancer patient sera. The incorporation of cell lines in this study provides confidence that the glycan repertoire

mimics that of human patients. This will be important for future experiments on the biological functions of aberrant glycosylation in breast cancer.

A3FG1 as a useful marker of metastasis and advanced breast cancer

The level of the sLex glycan marker was quantified, in the form of the digestion product A3FG1, in advanced breast cancer patients and controls (Figure 4). Our results indicate that breast cancer patients have on average a 2-fold increased level of sLex in the serum compared to controls. In 10 advanced breast cancer patients, we show that increased levels of this glycan marker may be a better indicator of metastasis and breast cancer progression in individual patients than CA 15-3. Although this preliminary finding requires further validation using a larger sample set, there have been various reports that support the usefulness of serum sLex evaluation in breast cancer. Measurement of serum sLex was previously carried out by Kurebayashi et al. (2006) using a radioimmunoassay (RIA) kit Fh6-Otsuka (Otsuka Assay Laboratory) with a cut-off value of 38 U/mL. In this study when sLex was used in combination with CA 15-3, an increased number of cases was detected: 78.5% compared to 61.5% CA 15-3 on its own, or 72.3% for the combination of CA 15-3 with CEA (Kurebayashi et al. 2006).

Similarly, high serum sLex has been shown to predict multilevel N2 stage and poor outcome of non-small cell lung cancer (NSCLC) and has been suggested as a useful staging marker in this disease (Mizuguchi et al. 2006). Serum sLex has also been shown to correlate with the soluble form of its ligand, E-selectin, in advanced and recurrent breast cancer (Matsuura et al. 1997).

Our group has also reported increased sLex in the serum of advanced ovarian (Saldova et al. 2007), lung (Arnold et al., in preparation), and prostate cancer (Tabares et al. 2006), as well as inflammatory conditions such as sepsis and pancreatitis (Gornik et al. 2007). Although these results rule out the usage of serum sLex in diagnosis and as a screening tool for breast cancer or any other malignancy, our results indicate that the derived glycan A3FG1, whose levels in serum reflect changes on many glycoproteins, can be used to monitor metastasis and breast cancer progression. The specificity of this marker is in agreement with conclusions reached by others that levels of sLex expressed on cell surface do not correlate with a specific disease (reviewed by Hakomori 2001). The levels of A3FG1 (which is mainly derived from sugars carrying the sLex epitope) do not reflect the patient's stage (individual levels in patients), but follow disease progression especially the onset of metastases. Therefore in our view, A3FG1 is most useful as a tool in personalized medicine for monitoring individual patients.

Altered glycosylation of acute phase proteins in breast cancer

In order to understand the biology behind the increased serum levels of sLex, it is crucial to determine the proteins carrying this structure. The acute-phase proteins, α1-acid glycoprotein, α1-antichymotrypsin, and haptoglobin β-chain (Hap) have all previously been reported to be present at elevated levels in breast cancer and may be indicative of metastases (Thompson et al. 1983). Following this, studies on the glycosylation of this group of proteins have revealed complex glycan structures with the sLex epitope (Brinkman-van der Linden et al. 1998). We identified the sLex glycan directly from these proteins present in breast cancer serum separated by 2D electrophoresis followed by immunoblotting and quantitative glycan analysis (Figure 6A).

AGP is classified as a positive acute-phase reactant and has five potential *N*-glycosylation sites, making it one of the most heavily glycosylated serum proteins. The biological role of AGP as anti-inflammatory in diseases is due to the sLex structure that it carries. High levels of sLex on AGP interfere with the selectin-mediated endothelial-leukocyte adhesion when E-selectin expression is enhanced by proinflammatory cytokines (De Graaf et al. 1993). Similarly in cancers, high concentrations of AGP carrying sLex results in a higher level of binding to E-selectin on endothelial cells which competes with cell surface sLex. This supports the hypothesis that circulating sLex exerts a feedback inhibitory effect on the extravasation of cancer cells, resulting in a defense mechanism against metastasis. The concept of inhibiting the sLex–E-selectin interaction for therapeutic design was employed by Fukami et al. (2002) who showed that metastasis could be suppressed by using Macrospelide B, which blocks sLex binding to E-selectin.

The work of Havenaar and co-workers (Havenaar et al. 1998) suggested that estrogen may affect AGP glycosylation by influencing the expression of cytokine genes which act on the liver glycosylation machinery. AGP and ACT have also been shown to be synthesized by human breast epithelial cells and are present at increased levels in MCF-7 culture media (Gendler et al. 1982). This supports the possibility that aberrant forms of both AGP and ACT might come from the breast tumor and not solely from the liver, as is generally accepted. This is also strengthened by the fact that breast cancer cells express the required glycosyltransferases (Matsuura et al. 1998) to produce altered glycoforms of AGP and ACT. Furthermore, ACT is an estrogen-inducible gene and its mRNA expression has been found useful for predicting early tumor recurrence in invasive breast cancer patients (Yamamura et al. 2004).

Haptoglobin is an iron transporter and has the highest binding affinity for hemoglobin. Fucosylated haptoglobin was increased in cancer serum compared to healthy controls (Thompson and Turner 1987) and correlated with response to therapy and tumor burden in breast as well as in ovarian cancer patients (Thompson et al. 1991).

Summary

In conclusion, we have sequenced the *N*-glycans of serum proteins from advanced breast cancer patients using a combination of NP- and WAX-HPLC, exoglycosidase digestion arrays and mass spectrometry techniques. This initial study has highlighted a significant increase in siaylation and changes in fucosylation in breast cancer patient sera compared to that from controls. More importantly, elevated levels of the sLex-carrying triantennary structure, A3FG1, derived from the monofucosylated trisialylated triantennary *N*-glycan (A3FG3S3), were detected in the serum of patients. We have evaluated the levels of this marker in a group of 18 patients and 18 controls and found an increase of more than 2-fold in advanced breast cancer. In a retrospective study on 10 breast cancer patients, we compared levels of this glycan marker to CA 15-3 at initial diagnosis and metastasis and observed that it serves as a more sensitive tool compared to CA 15-3 for detecting metastasis and breast cancer progression in general.

Our approach of combining serum proteomics with glycan analysis has highlighted the target proteins contributing to increased levels of this glycan marker, all of which were found to be acute-phase proteins. In a pilot longitudinal study performed on samples from an individual patient, the quantification of this glycan marker from these specific proteins proved to be better indicators of metastasis and disease progression, compared to CA 15-3. Our preliminary findings support the theory that inflammation is a key step in cancer progression and may provide additional targets for therapeutic intervention.

Taken together, this preliminary study has highlighted a specific glycan marker and a quantitative approach to monitor its level in breast cancer patients which we propose would be most beneficial as a tool in personalized medicine. This work warrants further investigations on the nature of aberrant glycosylation in breast cancer, not only in the search for an improved biomarker, but also, to improve our understanding of the disease.

Material and methods

Serum samples

Serum samples were obtained from cancer-free female controls and advanced breast cancer patients in the Breast Surgery Unit, Nottingham City Hospital, with informed consent prior to sample collection. An additional pooled control comprising serum from over 30 individuals was obtained from the British National Health Service (NHS) as analyzed in Royle et al. (2008).

The first study comparing the glycosylation of advanced breast cancer patients and controls involved 18 controls and 18 patients with the average age of 41 $\pm$ 13 years (range 24–66) and 63 $\pm$ 13 years (range 39–83), respectively. Estrogen receptor was positive in 16 patients and levels of CA15-3 were above 35 U/mL in 14 patients. All patients were stage 4 with metastasis. From this group of subjects, we analyzed the various glycan peaks by HPLC and performed multivariate statistical analysis on nine controls and eight patients. We also quantified the levels of the elevated glycan marker in all 36 samples. In addition, the degree of sialylation of all 36 *N*-glycan pools was also measured by WAX-HPLC.

To evaluate the potential of the glycan marker to indicate metastasis, 10 patients were monitored retrospectively by comparing levels of the marker in both early (at the time of diagnosis) and advanced stage samples (several months from the time of diagnosis where metastases were initially detected, Table I). CA 15-3 and CEA were measured for each sample using the CA 15-3 and CEA automated assays (Bayer Centaur, Newbury, UK).

The glycoproteomics experiment was performed on three samples obtained from a breast cancer patient at significant points (0, 47, and 61 months) of the disease duration based on CA 15-3 levels and clinical observations. Initially, the patient was seen in the clinic with an 8 $\times$ 7 cm lump. She had preoperative radiotherapy followed by mastectomy for a 100 mm grade 2 tumor and 16 out of 20 lymph nodes were positive (stage 3). The patient took a course of postoperative chemotherapy (CMF) followed by Zoladex and Tamoxifen. She presented with further pain and rising tumor markers with no evidence of progression in bone scans, ultrasound, and X-rays, and 61 months following diagnosis, metastasis was diagnosed in the liver and pleura. She received chemotherapy and further endocrine agents prior to her death, 85 months following initial diagnosis.

N-Glycan release by the in-gel block method

Serum samples (5 μL) were subjected to the in-gel block method as previously described (Royle et al. 2008). Briefly, *N*-glycans were released from serum gel blocks or glycoprotein spots excised from 2D gels of serum by protein *N*-glycosidase F (PNGaseF) digestion (100 U/mL, EC 3.5.1.52, Roche Diagnostics GmbH, Mannheim, Germany) carried out at 37°C for 18 h. The extracted glycan pool was then subjected to 2-aminobenzamide (2AB) fluorescent labeling using the Ludger Tag 2AB kit (Ludger, Oxford, UK).

Glycan analysis by HPLC and mass spectrometry

The labeled *N*-glycans were subsequently analyzed by 3 h runs NP-HPLC using a TSK gel Amide-80 column with a 20–58% gradient of 50 mM ammonium formate, pH 4.4, versus acetonitrile. The system was calibrated using an external standard of hydrolyzed and 2AB-labeled glucose oligomers which formed a dextran ladder. WAX-HPLC analysis of the *N*-glycans was carried out using a Vydac 301VHP575 7.5 $\times$ 50 mm column (Royle et al. 2006). Experimentally determined reproducibility for the quantitation of the A3FG1 peak was found to be 9.47% relative standard deviation using three individually prepared, digested, and analyzed aliquots of serum from the same patient.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

The positive-ion MALDI-TOF mass spectra were recorded with a Micromass TofSpec 2E reflectron-TOF mass spectrometer (Waters MS Technologies, Manchester, UK) fitted with delayed extraction and a nitrogen laser (337 nm). The acceleration voltage was 20 kV; the pulse voltage was 3200 V; the delay for the delayed extraction ion source was 500 ns. Samples were prepared by adding 0.5 μL of an aqueous solution of unlabeled glycans to the matrix solution (0.3 mL of a saturated solution of 2,5-dihydroxybenzoic acid in acetonitrile) on the stainless steel target plate and allowing the mixture to dry at room temperature. The sample/matrix mixture was then recrystallized from ethanol (Harvey 1993).

Negative-ion ESI-MS and ESI MS/MS

Nano-electrospray mass spectrometry was performed with a quadrupole time-of-flight (Q-Tof) Ultima Global instrument (Waters MS Technologies, Manchester, UK). Unlabeled glycan samples in 1:1 (v:v) methanol:water containing 0.5 mM ammonium phosphate were infused through Proxeon (Proxeon Biosystems, Odense, Denmark) nanospray capillaries. The ion source conditions were temperature, 120°C; nitrogen flow 50 L/h; infusion needle potential, 1.2 kV; cone voltage 100 V; RF-1 voltage 150 V. The spectra (2 s scans) were acquired with a digitization rate of 4 GHz and accumulated until a satisfactory signal:noise ratio had been obtained. For MS/MS data acquisition, the parent ion was selected at low resolution (about 4 *m/z* mass window) to allow transmission of isotope peaks and fragmented with argon. The voltage on the collision cell was adjusted with mass and charge to give an even distribution of fragment ions across the mass scale. Typical values were 80–120 V (Harvey et al. 2008).

Glycan sequencing and exoglycosidase digestion

N-Glycan structures were assigned glucose units (GU) by comparison to the retention time of a standard dextran ladder. Further

sequencing and structure confirmation was based on sequential exoglycosidase digestions followed by NP-HPLC (Royle et al. 2006). Labeled glycans were digested with an array of enzymes at manufacturer's recommended concentrations in a 50 mM sodium acetate buffer, pH 5.5, at 37°C for 16 h. The enzymes include *Arthrobacter ureafaciens* sialidase (ABS, EC 3.2.1.18), bovine testis β-galactosidase (BTG, EC 3.2.1.23), *Streptococcus pneumoniae* β-galactosidase (SPG, EC 3.2.1.23), almond meal α-fucosidase (AMF, EC 3.2.1.111), recombinant *Streptococcus pneumonia* hexosaminidase (GUH, EC 3.2.1.30), and jack bean β-*N*-acetylhexosaminidase (JBH, EC 3.2.1.30) purchased from Prozyme (San Leandro, CA) and Glyko (Novato, CA).

Two-dimensional gel electrophoresis of breast cancer serum

The 2D separation of the pooled control and breast cancer patient serum sample was carried out in duplicate for both anti-sLex blotting and fluorescent staining. Eighty micrograms of serum protein was used per gel based on the protein concentration determined by the Bicinchoninic acid (BCA) assay method of Smith et al. (1985). Each aliquot of serum was mixed with 5 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 65 mM dithiothreitol (DTT), 2 mM tributyl phosphine (TBP), 150 mM NDSB-256 (dimethylbenzylammonium propane sulfonate, nondetergent sulfobetaine-256-NDSB-256, Merck Biosciences Nottingham, UK), and 0.002% (w/v) bromophenol blue, 0.45% (v/v) of pH 2–4 carrier ampholytes (SERVALYT® SERVA, Heidelberg, Germany), 0.45% (v/v) of pH 9–11 carrier ampholytes, and 0.9% (v/v) of pH 3–10 carrier ampholytes, for a total volume of 120 μL per gel and transferred into reswelling trays. IPG strips (Immobiline® IPG DryStrip pH 3–10 NL, 7 cm; GE Healthcare, Uppsala, Sweden) were placed face down onto the samples, covered with 1 mL of mineral oil and left overnight at room temperature to allow rehydration (Sanchez et al. 1997).

Following this, the strips were transferred to the Multiphor II with the gel facing upward and damp wicks were placed on both ends. Isoelectric focusing (IEF) was carried out at 300 V for 1 min, 3500 V for 90 min, and then another 100 min at 3500 V (Sanchez et al. 1997). The IPG strips were then immediately equilibrated for 15 min in 4 M urea, 2 mM thiourea, 12 mM DTT, 50 mM Tris (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 30% (w/v) glycerol at room temperature and placed on top of the second dimension 4–12% Bis-Tris Zoom (Invitrogen, Carlsbad, CA) gels embedded in 0.5% melted agarose. Second dimension electrophoresis was carried out at 125 V for 2 h. A gel from each sample was fixed in 40% (v/v) ethanol, 10% (v/v) acetic acid overnight, and stained with the fluorescent dye OGT 1238 (Oxford Glycosciences, Abingdon, UK) according to Hassner et al. (1984). Eight-bit monochrome fluorescent images were captured at using a FujiCCDC Camera LAS_1000 plus (Tokyo, Japan).

For immunoblotting, proteins were transferred to a nitrocellulose membrane. Membranes were blocked with 0.2% (w/v) casein (I-Block, Tropix, Bedford, MA) in phosphate-buffered saline Tween (PBST) for 1 h at room temperature before an overnight incubation in 5 μg/mL KM93 (Calbiochem, Nottingham, UK) in the 0.02% (w/v) blocking solution at 4°C. Membranes were washed with 0.5% PBST before 1 h incubation with 0.5 μg/mL anti-mouse immunoglobulin M (IgM) (Sigma, Dorset, UK). The blots were developed using the ECL Plus chemiluminescent detection system (GE Healthcare, Uppsala, Sweden).

N-Glycan release, peptide extraction, liquid chromatography MS/MS (LC-MS/MS) and data analysis for protein identification

Protein features assigned for mass spectrometric analysis were excised manually. The recovered gel pieces were reduced with 0.5M DTT at 65°C for 20 min followed by a 30-min incubation in 100 mM iodoacetamide (IAA) and an overnight digestion with PNGaseF to cleave the *N*-glycans, as described earlier. Following glycan extraction, the gel pieces were dried in a vacuum vaporizer, and in-gel trypsin digestion was carried according to the protocol of Shevchenko et al. (1996) using sequencing grade bovine trypsin (Roche, East Sussex, UK).

Mass spectrometric analysis was carried out using a Q-TOF 1 (Waters MS Technologies, Manchester, UK) coupled to a CapLC (Waters, Milford, MA) HPLC system. Tryptic peptides were concentrated and desalted on a 300 μm i.d., 5 mm C18 precolumn, and resolved on a 75 μm i.d., 25 cm C18 PepMap analytical column (LC Packings, SanFrancisco, CA). Peptides were eluted to the mass spectrometer using a 45-min 5–95% acetonitrile gradient containing 0.1% formic acid at a flow rate of 200 nL/min. The spectra were acquired in positive mode with a cone voltage of 40 V and a capillary voltage of 3300 V. The MS to MS/MS switching was controlled in an automatic data-dependent fashion with a 1-s survey scan followed by three 1-s MS/MS scans of the most intense ions. Precursor ions selected for MS/MS were excluded from further fragmentation for 2 min. The spectra were processed using ProteinLynx Global server 2.1.5 and searched against the SWISS-PROT and NCBI databases using the MASCOT search engine (Matrix Science, London, UK).

Searches were restricted to the human taxonomy allowing carbamidomethyl cysteine as a fixed modification and oxidized methionine as a potential variable modification. Data were searched allowing 0.5 Da errors on all spectra and up to two missed tryptic cleavage sites to accommodate calibration drift and incomplete digestion; all data were checked for consistent error distribution, and positive identifications were checked manually.

Statistics

The one-sample Kolmogorov–Smirnov test was carried out to determine normality of data distribution in each group. A two-tailed Mann–Whitney *U*-test was used for a comparison of data between non-normally distributed groups, and Student's two-tailed *t*-test for independent groups was applied in cases of normal distribution. This was performed using SPSS 12.0.1 software (Dublin, Ireland). Multivariate statistics (PLS-DA) and importance of variable (individual peaks) plots were generated by using the SIMCA-P+ 12 software (Umetrics, Ascot, UK).

Funding

The Oxford Glycobiology Institute Endowment fund and EUROCarbDB (http://www.eurocarbdb.org) RIDS (Contract No. 011952) and the Malaysian Ministry of Higher Education and MARA University of Technology (U.M.A.H.).

Acknowledgements

The authors are grateful to Dr. Bevin Gangadharan for advice on proteomic analysis, Ms. Jodie Abrahams for the IgG glycan standard, Dr. Jonathan Bones for technical advice, and Dr. Marion Boland for careful reading of this manuscript. They also thank the Biotechnology and Biological Sciences Research Council and the Wellcome trust for grants to purchase the Tof-Spec and Q-Tof mass spectrometers, respectively.

Conflict of interest statement

None declared.

Abbreviations

2AB, 2-aminobenzamide; 2D, two-dimensional; ABS, *Arthrobacter ureafaciens* sialidase; ACT, α1-antichymotrypsin; AGP, α1-acid glycoprotein; AMF, almond meal α-fucosidase; BCA, bicinchoninic acid; BTG, bovine testis β-galactosidase; CA, cancer antigen; CD, cluster differentiation; CEA, carcinoembryonic antigen; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; dHex, deoxyhexose (fucose); DTT, dithiothreitol; ESI, electrospray ionization; Fuc, fucose; FucT, fucosyltransferase; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GnT, *N*-acetylglucosaminetransferase; GU, glucose unit; GUH, β-*N*-acetylglucosaminidase cloned from *Streptococcus pneumoniae*, expressed in *E. coli*; Hap, haptoglobin; Hex, hexose; HexNAc, *N*-acetylhexosamine; HPLC, high-performance liquid chromatography; IAA, iodoacetamide; IEF, isoelectric focusing; IgM, immunoglobulin M; IPG, immobilized pH gradient; JBH, jack bean β-*N*-acetylhexosaminidase; LC, liquid chromatography; Lex, Lewis x; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; NDSB, nondetergent sulfobetaine; NHS, National Health Service; NK, natural killer; NP, normal phase; NSCLC, nonsmall cell lung cancer; PAGE, polyacrylamide gel electrophoresis; PBST, phosphate-buffered saline Tween; PLS-DA, Partial Least Squares-Discriminant Analysis; PNGase F, protein *N*-glycosidase F; PSA, prostate-specific antigen; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; sLex, sialyl Lewis x; SPG, *Streptococcus pneumoniae* β-galactosidase; ST, sialyltransferase; TBP, tributyl phosphine; TOF, time-of-flight; WAX, weak anion exchange.

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