Cancer Biology

Elevated level of serum glycoprotein bifucosylation and prognostic value in Chinese breast cancer

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Received 31 October 2014; Revised 25 November 2015; Accepted 30 November 2015

Abstract

Aberrant glycosylation is highly associated with cancer progression. The aim of this study was to compare bifucosylated N-glycans in sera obtained from healthy controls and breast cancer patients, with the goal of identifying a potential indicator for monitoring the recurrence and metastasis of breast cancer. A unique structural pattern of bifucosylated N-glycan, with both core and antennary fucosylation, was identified in breast cancer patients. The spectrum of antennary fucosylation was a composite of the standard spectra of Lewis X and H2, indicating a mixture of the two epitopes. Permethylated N-glycans of the glycoproteins extracted from 91 breast cancer patients and 43 healthy controls were detected using linear ion-trap quadrupole-electrospray ionization mass spectrometry, which appeared to be a highly sensitive and useful approach in the detection and identification of N-glycans. To evaluate MS profile data, several statistical tools were applied, including Student’s t-test, partial least squares discriminant analysis and receiver-operating characteristic curve. The results showed that the measurement of bifucosylation degree and CEA levels had an improved diagnostic performance compared with that of CEA alone. We compared the potential of bifucosylated N-glycan as an indicator of breast cancer recurrence with the current clinical biomarkers, i.e., CEA, CA 15-3 and CA125. The result revealed that, compared with CEA, CA 15-3 and CA125, the bifucosylation degree of N-glycans could be a more reliable indicator of breast cancer recurrence.

Key words: bifucosylation, breast cancer, mass spectrometry, N-glycan

Introduction

Breast cancer, the most prevalent cancer worldwide, accounts for the highest number of cancer-related deaths in women. It has been reported that early detection and diagnosis significantly improve the survival rate of breast cancer patients (Liu et al. 2013; Debald et al. 2014). Currently, breast cancer is diagnosed by measuring (Takegawa et al. 2005) levels of estrogen receptors, progesterone receptors, human epidermal growth factor receptor 2, and Ki67 (Weigel and Dowsett 2010; Cole et al. 2013; Margalit et al. 2013; Hu et al. 2014). However, these diagnostic approaches are not sufficiently sensitive and specific. Therefore, there is an urgent need to find reliable biomarkers of breast cancer.

Glycosylated proteins play important roles in several biological functions including cell–cell interactions, signal transduction and
receptor-mediated and protein functions (Kyselova et al. 2008). More than 45 years of investigation have shown that nearly all human cancers have aberrant glycosylation patterns (Hakomori 2002). The current panel of tumor biomarkers, e.g., CA 15-3 and CEA in breast cancer, prostate-specific antigen in prostate cancer, CA19-9 in pancreatic cancer, AFP in hepatic cancer and CA-125 in ovarian cancer, are glycoproteins with altered glycans profiles (Abd Hamid et al. 2008; Whelan et al. 2009). Aberrant glycosylation of glycoproteins that are either expressed or secreted by cancer cells is correlated with increasing tumor burden and poor prognosis (Abd Hamid et al. 2008). N-Glycans of serum proteins from advanced breast cancer patients and healthy controls were sequenced by HPLC with fluorescence detection coupled with exoglycosidase digestions and mass spectrometry. Abd Hamid also found that a significant increase in a triasiallylated triantennary glycan containing α1,3-linked fucose which forms part of the sialyl Lewis x epitope.

Along with genomics and proteomics, glycomics has garnered considerable attention. Glycosylation changes of serum proteins, which are closely associated with breast cancer progression, have been explored as diagnostic indicators of breast cancer (Kyselova et al. 2008; Potapenko et al. 2010). The total glycosylation level of serum proteins was elevated from Stage I to Stage IV breast cancer patients based on MALDI/MS of quantitatively permethylated N-glycans. Potapenko also found that an increase in core fucosylation of N-glycans for the circulating α1-proteinase inhibitor in breast cancer patients. The mRNA transcript for FUT8 gene was up-regulated in carcinomas indicating higher contents of complex glycans in tumors.

In this study, we analyzed serum N-linked glycans in breast cancer patients and healthy controls using LTQ-ESI-MS. LTQ-ESI-MS is particularly useful in generating ions from macromolecules, because it overcomes the propensity of these molecules to defragment when ionized (Han and Gross 2003; Schudde et al. 2006). The direct application of ESI-MS maintains the structural integrity of N-glycans and allows the potential identification of these compounds. Using multistage MS (MS^n), we analyzed the structure of N-glycans from both groups. Finally, we analyzed 40 N-glycans using two-tailed Student’s t-test and receiver-operating characteristic (ROC) analysis. Our preliminary findings suggest that N-glycan bifucosylation was significantly higher in breast cancer patients compared with healthy controls. Increased bifucosylation of glycan structures appears to be indicative of cancer progression.

**Results**

Several tumor-specific glycoconjugates have been targeted as antigens in breast cancer, including Globo-H (Wang et al. 2008), CA153 (Cheung et al. 2000; Duffy 2006), MUC1 Tn (Cheung et al. 2000; Baldus et al. 2005) and CEA (Weigel and Dowsett 2010); however, none of these compounds are reliable serum biomarkers of cancer. Therefore, the purpose of this study was to assess differences in N-glycan levels and structure in sera from breast cancer patients and healthy controls. Additionally, we investigated aberrant glycosylation of N-glycans in initial diagnostic and recurrent breast cancer patients. Changes in N-linked bifucosylation have been highly associated with breast cancer progression.

**Glycomic profiles derived from human serum**

LTQ-ESI-MS analyses of permethylated N-glycans, which were enzymatically detached from serum glycoproteins, allowed the differentiation of N-glycan structures derived from sera of healthy controls (n = 43) and breast cancer patients (n = 91). The 91 breast cancer patients included initial diagnostic patients (n = 47) and recurrent breast cancer patients, who had undergone chemotherapy (n = 44). From the MS1 profile spectrum of the permethylated fraction, >40 ions were detected in the two sample sets, with each peak representing a different N-glycan. As shown in Figure 1, the profiles of permethylated N-glycans derived from healthy controls (A), initial diagnostic patients (B) and recurrent breast cancer patients (C) were recorded in the m/z range of 800–1500. Only representative profiles are illustrated in Figure 1. The profiles were different among the three sample sets. Most of the ions in the MS1 profile spectrum of N-glycans were doubly charged, and all the ions were sodium adducts.

To further confirm the structure of the N-glycans, we used sequential multistage MS to compare their linkage and branching characteristics. All ions were confirmed to be glycans by MS^n analysis. The N-glycan structures are presented in Supplementary data, Table S1. The bifucosylated N-glycan composition and core/antennary fucosylation were further confirmed by ESI-MS/MS analysis (Supplementary data, Figure S1). The ion at m/z 1453²⁺, corresponding to N5H6F2, was isolated from each of the profiles and analyzed by ESI-MS (Figure 2).

Figure 2 shows the MS^n of Fuc-HexHexNAc at m/z 1453²⁺, which followed the MS^n pathway of m/z 1453²⁺→1221²⁺→990²⁺→1319→852→648 (Figure 2A–F). In the MS² spectra (Figure 2A), the examination of these product ions revealed three matched B-ion/Y-ion complements: (i) m/z 486.3/m/z 1121.7²⁺; (ii) m/z 490/m/z 1219.7²⁺; and (iii) m/z 660.4/m/z 1134.7²⁺. The ion fragments at m/z 486 (B2-ion) represented terminal Gal-GalNAc, the ion at m/z 490 corresponded to a fucosylated reducing-end GlcNAc, the ion at m/z 660 was consistent with a B1-ion consisting of fucose, galactose and glucosamine residues (Supplementary data, Table SI). The most intense ions in the spectrum (MS²+) resulted from the loss of terminal Hex-HexNAc disaccharides. However, MS³ revealed an additional loss: terminal Fuc-Gal-GalNAc–B1 ions. To confirm such product, the ion at m/z 648 was analyzed by MS^n. The results revealed a unique signature fragment as the base ion (m/z 444.0; Figure 2F), indicating a bisected GlcNAc. The core fragment, m/z 444, Man-GlcNAc with four scars (3 on the Man), was a product of a number of pathways identifying a bisected substructure. The ion at m/z 1453 has an unusual β1–4 bisecting branch (Galβ1–4 bisecting GlcNAc). Takegawa et al. (2005) reported the structural analysis of a similar N-glycan with “β1–4 bisecting branch” (galactose (Gal) β1–4 bisecting N-acetylgalcosamine (GlcNAc)) in human IgG. To confirm whether a galactose is in fact transferred to a bisecting GlcNAc, they used β1–4 galactosyltransferase to a reaction of the bisecting N-glycan. Thus, the conclusion is that the N-glycan is a galactosylated product of the bisecting N-glycan. Because β1–4 galactosyltransferase specifically transfers a galactose (Gal) to GlcNAc with β1–4 glycoside bond (i.e., Gal β1–4 GlcNAc) and the other two GlcNAc residues on the R1–6 and R1–3 branches are already galactosylated, it is reasonable to think that this galactosylation occurred on the bisecting GlcNAc residue (Takegawa et al. 2005).

The m/z 660 ion had several isomers. We identified the m/z 660 fragment from bifucosylated N-glycans via ESI-MSn among 90 samples. Lewis X, Lewis A, and H antigens are isomeric and contain the m/z 660 ion. The Lewis X standard spectrum showed a small but important fragment ion at m/z 329, representative of a 3,5A cross-ring cleavage, which places the hexose at either the 4- or 6-position and clearly distinguishes Lewis X from Lewis A. The Lewis A standard spectrum contains a significant peak at m/z 442, representing the loss of a terminal hexose residue, because neutral loss of the 3-linked...
substituent is highly favored over the 4-linked substituent in these iso-
mers (Supplementary data, Figure S2A and B). H antigens have similar
substructure mass; however, they are easier to distinguish than Lewis
X and Lewis A, because fucose is attached to galactose rather than to
glucosamine. H antigens are based on Type 1 (Gal-β1,3-GlcNAc) or
Type 2 (Gal-β1,4-GlcNAc) lactosamine. The fragment at m/z 503 in
the H type 2 spectrum is a 3,5-A cleavage, which places the Fuc-Gal
moiety at the 4-position in GlcNAc. This fragment was absent from
H type 1, and m/z 503 was specific to H type 2 (Supplementary
data, Figure S2C and D). We treated N-glycans with α1,2 fucosidase,

an enzyme that hydrolyzes the terminal fucose residues linked to gal-
actose via α1,2 linkage. The intensity of ions at m/z 415, m/z 433 and
m/z 503 in the MS5 spectra decreased following treatment with α1,2
fucosidase. The terminal α1,2 linkage for Fuc-Gal was con-
firmed following fucosidase addition (Supplementary data, Figure S3). The
structure of the m/z 660 fragment was based on antennal identi-
fication developed by Ashline et al (Ashline et al. 2014). The m/z 660 spectrum
is a composite of the standard spectra of Lewis X and H2. The bifu-
cosylated glycans (m/z 1228, 1352, 1409 and 1453 ions) have the
same epitopes (Supplementary data, Figure S4).

Fig. 1. Representative ESI mass spectra of N-glycans derived from a 50-µL serum aliquot of healthy controls (A), initial diagnostic patients (B), and recurrent breast cancer patients (C).
Fig. 2. MS²^− pathways of the ion at m/z 1452 (doubly charged) in a breast cancer patient sample. Pathways appear as inserts in the top right corner of the spectrum. Fragments accounting for the spectrum are delineated by dotted lines. Mannose: black solid circle; Gal: black hollow circle; Fuc: black triangle; GlcNAc: black squares.
Mass spectrometry data for partial least squares discriminant analysis

PLS-DA is widely used to construct a multivariate discrimination model in spectral analyses. PLS-DA projects both the predicted and obtained variables into a new space to maximize the covariance between the response and independent variables (So et al. 2013; Nishiki-Muranishi et al. 2014). PLS-DA was applied to process the acquired and normalized data using SIMCA-P+ software (version 12, Umetrics, Umeå, Sweden). The calculated R²Y (cum) estimates the goodness of fit of the model that represents the fraction of explained Y-variation, and Q² (cum) estimates the ability of prediction (Zhang et al. 2014).

Based on the percentages of the N-glycan peaks, we obtained a clear discrimination between sera from breast cancer patients and healthy controls (Figure 3A). The PLS-DA model was constructed with four significant PLS components resulting in an excellent separation R²Y (cum) of 98.5% and a good prediction ability Q² (cum) of 97.6%; a reliable model has R²Y and Q² cumulative values >0.8 (Zhang et al. 2014).

The variable importance in the projection (VIP) value reflects the relative contribution of each X variable to the model. VIP values >1.0 indicate important X variables, which have significant effects on group distinction (Holst et al. 2013; Urbach et al. 2014; Zhang et al. 2014). The VIP values of the 40 N-glycan peaks are presented in Figure 3B.

Comparative analyses of major aberrant N-glycans

To further investigate the aberrant expression of N-glycans in breast cancer patients, we compared the percentage of N-glycans (mean ± SD) between healthy controls and breast cancer patients. Analysis of the ion relative intensities by Student's t-test revealed significant differences in the abundance of 11 N-glycans. As shown in Figure 4, the expression of 11 N-glycans was significantly (P < 0.05) higher in breast cancer patients than in healthy controls. These results are in agreement with the VIP values of the PLS-DA model. We analyzed the structure of the 11 N-glycans. Peaks at m/z 809, 1013, 1218 and 1320 corresponded to high-mannose N-glycans, while peaks at m/z 815, 1040, 1127, 1228, 1352, 1409 and 1453 corresponded to fucosylated N-glycans. In addition, peaks at m/z 1127, 1228, 1352, 1409 and 1453 were attributed to bifucosylated N-glycans. Notably, these five bifucosylated N-glycans were significantly higher in breast cancer samples compared with the other seven N-glycans (P < 0.001; Figure 5A–D).

Fig. 3. Overview of the PLS-DA plot and importance of variable (individual peaks) plots of ESI-LTQ-MS N-glycans in breast cancer patients versus healthy controls. (A) Score plot showing the separation of samples from breast cancer patients (red circles) and healthy controls (black squares) based on the information obtained from the MS profiles. (B) The VIP value reflects the relative contribution of each X variable (N-glycan) to the model. This figure is available in black and white in print and in color at Glycobiology online.
Bifucosylation degree increased in sera from breast cancer patients. This result suggests that bifucosylation degree was a good diagnostic method to distinguish samples from the two groups.

A further statistical analysis of aberrant N-glycans was performed by ROC. Figure 5F shows the ROC analysis of five bifucosylated N-glycans (peaks at m/z 1127, 1228, 1352, 1409 and 1453). AUC of ions at m/z 1127, 1228, 1352, 1409 and 1453 was 0.789, 0.854, 0.844, 0.826 and 0.764, respectively. A cumulative AUC value was obtained from the sum of these five bifucosylated N-glycans. The closer AUC is to 1, the better the overall diagnostic performance of the test. The test is considered to be highly accurate at AUC values ≥ 0.9 (Pepe et al. 2006; Kyselova et al. 2007). Interestingly, the cumulative AUC value was 0.913. ROC analysis of high-mannose levels in healthy controls and breast cancer patients is shown in Supplementary.

![Fig. 4. Relative intensity changes of 11 aberrant N-glycans in samples from healthy controls and breast cancer patients. A Student’s t-test comparison was performed between the two groups. Error bar indicates SD (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).](image)

![Fig. 5. Scatter plot of the expression levels of five bifucosylated N-glycans in healthy controls and breast cancer patients. Bifucosylation levels were significantly higher in breast cancer patients than in healthy controls. (A) m/z 1127, (B) m/z 1228, (C) m/z 1352, (D) m/z 1409 and (E) m/z 1453. (F) ROC curve analysis of the bifucosylated N-glycans. The ion at m/z 1127 (blue line) had an AUC value of 0.789; the ion at m/z 1228 (green line) had an AUC value of 0.854; the ion at m/z 1352 (gray line) had an AUC value of 0.844; the ion at m/z 1409 (purple line) had AUC value of 0.826; and the ion at m/z 1453 (yellow line) had an AUC value of 0.764. Total bifucosylation levels (red line) had an AUC value of 0.913. Statistical significance was determined by two-tailed Student’s t-test (***P<0.001; ****P<0.0001).](image)
the expression of high-mannose N-glycans was higher in breast cancer patients than in healthy controls; however, due to their low sensitivity and specificity, they have no value in breast cancer detection (Supplementary data, Figure S5). On the other hand, the five bifucosylated N-glycans had high specificity and sensitivity in breast cancer detection.

**Combinatorial analysis of bifucosylation degree with CEA**

Sera from individuals with different types of carcinomas including breast cancer have higher levels of CEA than healthy controls. As a result, CEA is frequently used as a breast cancer biomarker (Lee et al. 2013a; Park et al. 2014; Wu et al. 2014). Because the bifucosylation degree of N-glycans was distinctly elevated in breast cancer patients compared with that in healthy controls, we performed a 2D plot of the bifucosylation degree and CEA levels between the two groups. As shown in Figure 6A, most breast cancer patients (represented by red circles) were in the upper panel of the plot, indicating high levels of bifucosylation degree. In contrast, the healthy controls (represented by blue circles) were clustered in the lower left panel of the plot, indicating low levels of both bifucosylation degree and CEA levels.

We investigated the diagnostic capacity of bifucosylation degree and CEA in breast cancer detection by comparing the parameters from ROC curve analysis. Bifucosylation degree and CEA had a combined AUC value of 0.963, showing a distinctively improved diagnostic performance compared with that of CEA alone (AUC = 0.794; P < 0.0001). However, there was no statistical difference in CEA levels between initial diagnostic patients and recurrent breast cancer patients (P = 0.1954; Figure 8C). Serum CA 15-3 levels in recurrent breast cancer patients was slightly elevated as a function of age; however, the result was not statistically significant (P = 0.0228). This preliminary result suggests that, compared with CEA, CA 15-3 and CA125 levels, bifucosylation degree can be a more reliable indicator of breast cancer recurrence. Consequently, bifucosylated N-glycans might play a role during tumorigenesis. Based on this observation, we speculate that these bifucosylated N-glycans can be used to monitor the recurrence and metastasis of breast cancer.

**Relationship between bifucosylation degree and age**

The five bifucosylated N-glycans were obtained from sera of 91 breast cancer patients who were 32–76 years of age. Figure 9A shows that bifucosylation degree was slightly elevated as a function of age; however, the result was not statistically significant (P = 0.1317). Similarly, in healthy controls (25–76 years of age), there was no association between bifucosylation degree and age (P = 0.8563; Figure 9B).

**Discussion**

Alterations in N-linked glycosylation in cancer and other chronic diseases have gained a lot of research interest. The analysis of structural N-glycan features may potentially identify specific glycans involved in tumorigenesis, unveil unique cancer biomarkers and discover novel targets for tumor immunotherapy. Ion-trap MSn represents a suitable method for detecting detailed glycosylation changes and patterns in breast cancer patients.

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**Fig. 6.** (A) Two-dimensional plot of bifucosylation degree and CEA levels in healthy controls and breast cancer patients. Each spot represents an individual sample. (B) ROC curve analysis of CEA levels and bifucosylation degree. CEA (blue line) had an AUC value of 0.794. Bifucosylation degree (green line) had an AUC value of 0.913. Bifucosylation degree and CEA levels (red line) had a combined AUC value of 0.963. This figure is available in black and white in print and in color at Glycobiology online.
Low bifucosylated N-glycan levels were detected in healthy controls. On the other hand, bifucosylated N-glycan levels were high in initial diagnostic and recurrent breast cancer patients. Bifucosylated N-glycans, with both core and antennary fucosylation, were predominantly present in recurrent breast cancer patients, suggesting that this glycan may be associated with the prognosis of breast cancer patients.

Lewis antigens are functionally important terminal glycan epitopes, which were first reported to be implicated in breast cancer development several decades ago (Fukushima et al. 1984; Potapenko et al. 2010). Lewis antigens comprise type 1 (Lewis A and Lewis B) and type 2 (Lewis X and Lewis Y) carbohydrates. Lewis X and Lewis Y are considered to be tumor-associated markers, and some of these antigens and their derivatives interact with selectins, mediating cell-to-cell adhesion (Soejima and Koda 2005). Lewis epitopes, particularly in Lewis X, are upregulated in various types of cancers. The expression of Lewis X in certain breast cancer cell lines (Elola et al. 2007) is upregulated and associated with poor prognosis of breast cancer patients (Koh et al. 2013). Koh et al. reported that Lewis X expression is an independent prognostic factor for recurrence-free survival and overall survival in young patients with triple-negative breast cancer types. Lewis X may play a role in the interaction between breast carcinoma cells and endothelial cells promoting metastases, although presumably through a different mechanism (Elola et al. 2007; Potapenko et al. 2010). Lewis Y is a doubly fucosylated oligosaccharide. As a cancer-associated antigen, Lewis Y has received considerable attention because of its role in the genesis and development of tumors.

Fig. 7. Scatter plot of five bifucosylated N-glycans in healthy controls, initial diagnostic patients and recurrent breast cancer patients. (A) $m/z$ 1127, (B) $m/z$ 1228, (C) $m/z$ 1352, (D) $m/z$ 1409 and (E) $m/z$ 1453. (F) Changes in five bifucosylated N-glycans from sera of healthy controls and breast cancer patients in two different stages. Statistical significance was assessed by two-tailed Student’s t-test (**$P<0.01$; ***$P<0.001$; ****$P<0.0001$).
Gao et al. reported that CD147 and Lewis Y expression levels and ovarian cancer stage are independent risk factors for drug resistance and prognosis in ovarian cancer (Gao et al. 2014). High expression of Lewis Y/B antigen is associated with decreased survival in lymph node-negative breast carcinomas (Madjd et al. 2005). H type 2 antigen is a fucosylated oligosaccharide with the chemical structure Fucα(1-2)Galβ(1-4)GlcNAc-R. Studies have reported that Fucα(1-2)Gal is characteristic of a malignant phenotype in several human breast cancers (Chang et al. 2008; Zhu et al. 2015).

In recent years, the prognostic value of CEA, CA15-3 and CA125 in breast cancer has gained considerable attention. The first tumor biomarker ever used for the diagnoses of different types of cancers was CEA, which is overexpressed in oncological patients (Mirabelli and Incoronato 2013). CA15-3, a member of the mucin-1 family of glycoproteins, is similarly overexpressed in cancer patients (Wu et al. 2014). CA125 is a tumor marker that has been commonly used in the diagnosis of ovarian cancer; however, CA125 levels may be elevated (up to 84%) in metastatic breast cancer patients (Yerushalmi et al. 2012). However, the diagnostic value of CEA, CA15-3 and CA125 in breast cancer is questionable.

Some researchers have reported that the expression of CEA, CA15-3 and CA125 has an important value in the prognosis, early
detection and treatment monitoring of breast cancer (Lee et al. 2013b; Thriveni et al. 2013; Zhang et al. 2013). In contrast, Maric et al. (2011), who reviewed the role of serum tumor markers in breast cancer, reported that there are conflicting results regarding the prognostic value of CEA, CA15-3 and CA125. In addition, there is evidence showing that these markers are not sufficiently sensitive or specific in the early diagnosis of breast cancer (Clinton et al. 2003; Mirabelli and Incoronato 2013; Park et al. 2014). In fact, the American Society of Clinical Oncology does not recommend the use of serum CEA and CA15-3 for breast cancer screening due to inconsistent findings (Mirabelli and Incoronato 2013; Wu et al. 2014). Therefore, further clinical trials based on tumor marker levels are required.

Aberrancy in glycan processing may lead to cancer-specific structural isomers. The analyses of these glycan structures might shed light on their role in tumor progression and their value in cancer detection.

**Conclusion**

Lectins and antibodies are used in the analysis of glycan epitopes; however, mass spectrometry offers several potential advantages. Specifically, mass spectrometry can provide evidence of isomeric mixtures, especially in biological samples that contain multiple positional isomers. The structure of bifucosylated N-glycans containing both core and antennary fucosylation can be determined by MS*. In this study, bifucosylated N-glycans contained Lewis X and H type 2, but not Lewis Y.

The MS-based glycan profile analysis of breast cancer sera revealed differences between healthy controls and breast cancer patients. In this study, we described a quantitative mass spectrometry-based approach to determine fucosylation alterations of serum proteins in 91 breast cancer patients. PLS-DA, ROC and Student’s t-test analyses independently confirmed that five bifucosylated N-glycans (P < 0.001) were significantly different between healthy controls and breast cancer patients. The results showed that bifucosylation degree was increased in breast cancer patients. The diagnostic capacity of CEA could be notably improved by measuring bifucosylation degree. Even though it is not yet clear which serum glycoproteins are responsible for the specific N-glycans, the measurement of aberrant N-glycans may be useful in the development of diagnostic procedures.

Bifucosylation degree was investigated in initial diagnostic and recurrent breast cancer patients. Bifucosylation degree was clearly elevated in recurrent breast cancer patients. Furthermore, there were no significant relationships between bifucosylation degree and age. Exploring variations in glycosylation may be a viable method in disease diagnosis. Additionally, N-glycans may be potential targets of drugs in tumor therapy (Liu et al. 2013). Meanwhile, knowledge of these specific N-glycans may help to understand the mechanisms of progression and metastasis of breast cancer.

**Material and methods**

**Serum samples**

In this study, 134 serum samples (91 from breast cancer patients and 43 from healthy controls) were provided by the Affiliated Hospital of Jiangsu University (Zhenjiang, China). The study was approved by the ethics committee of the Affiliated Hospital of Jiangsu University. Written informed consent was obtained from each participant. The characteristics of the participants are summarized in Table I. Serum aliquots were frozen immediately after collection and stored at −80°C.

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<td><strong>CA-153 (median, U/mL)</strong></td>
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**Release of N-glycans from glycoproteins**

A 50-µL aliquot of lyophilized serum was dissolved in 100 µL of 100 mM ammonium bicarbonate (NH₄HCO₃) in H₂O (water used in all experiments was filtered with the Milli-Q filtration system) and incubated with 40 µL of 10 mM dithiothreitol at 56°C for 45 min. After cooling to room temperature, the sample was incubated with 40 µL of 55 mM iodoacetamide in 100 mM NH₄HCO₃ at room temperature for 30 min in the dark. Subsequently, trypsin (dissolved in 50 mM NH₄HCO₃, PH 8.4, 10 µg/mL) was added to the solution at an enzyme/substrate ratio of 1:10 (w/w) and incubated at 37°C for 18 h. Trypsin was heat inactivated by boiling the solution for 10 min. Subsequently, the reaction mixture was lyophilized and diluted with 100 µL of ammonium bicarbonate to adjust the pH to 8.0–8.5. Peptide-N-glycosidase F (PNGase F, 5 mU) was added to the reaction mixture and incubated overnight (18–22 h) at 37°C.

**Solid-phase extraction of N-glycans**

Glycans were purified using Sep-Pak C18 cartridges (Waters), previously rinsed with 2 mL acetonitrile and equilibrated with 3 mL of 2% acetonitrile in 0.1% trifluoroacetic acid (TFA). Glycans were eluted with 1 mL of 2% acetonitrile (ACN) in 0.1% TFA, dried, reduced with sodium borohydride (NaBH₄, 10 mg/mL in 0.01 M NaOH) and left overnight at room temperature. The reaction mixture was placed in an ice bath and mixed with 20% acetic acid followed by 3 mL of ethanol. Samples were desalted using a porous graphitized carbon (PGC) column. The PGC column was cleaned three times with 3 mL of each of the following solutions, 1 M NaOH, water, 80% ACN in water with 0.1% TFA, 25% ACN in water with 0.05% TFA, 25% ACN and water. The glycans were dissolved in 1 mL water, loaded to the column and rinsed with water (6–8 mL) to remove impurities and salt. Neutral glycans were eluted with 3 mL of 25% ACN, and acidic glycans were eluted with 3 mL of 25% ACN with 0.1% TFA. The collected samples were dried under vacuum and permethylated.

**Permethylation of glycans**

Glycoprotein-derived N-glycans were permethylated (Ciuca and Costello 2003). Briefly, the glycans were dissolved in 150 µL of dimethyl sulphone in a conical glass vial. Sodium hydroxide (40–60 mg) and iodomethane (80 µL) were added, and the mixture was shaken vigorously for 1 h at room temperature. The permethylation reaction was stopped with the addition of 2 mL of water. Dichloromethane (2 mL) was added and mixed vigorously. The permethylated N-glycans were extracted with dichloromethane and rinsed repeatedly (10 times) with 2 mL of water. Following the final wash, the aqueous...
To further con, a tube lens of 165 V, an injection time of 100 ms, an activation time of 3.50 kV, a capillary voltage of 35 V, a capillary temperature of 275\degree C, a tube lens of 165 V, an injection time of 100 ms, an activation time of 30 ms, an activation Q-value of 0.250 and an isolation width of m/z 1.5. To further confirm the structure of N-glycans, MSn spectra were obtained. All samples were analyzed as sodium adducted positive ions.

**Data analysis**

To obtain the relative quantification of each N-glycan, we applied the following formula, (intensity of specific N-glycan peak)/intensity of total N-glycan peaks). Data were analyzed by two-tailed Student’s t-test using GraphPad Prism software (La Jolla, CA). Statistical significance was set at P < 0.05.

PLS-DA was performed to maximize sample separation of N-glycans from healthy controls and breast cancer patients. The VIP ranks the variables according to their significance in the model, allowing the identification of differential structures contributing to sample separation (Balog et al. 2012; Kang et al. 2014).

Additionally, we performed ROC analysis to assess the sensitivity and specificity of the potential diagnostic variables. ROC analysis was performed by SPSS software (Ver. 17.0; Chicago, IL, USA). AUC value represents the area under the curve in the ROC graph. ROC analysis and AUC calculations were performed to determine the sensitivity and specificity of different N-glycan markers.

**Supplementary data**

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

**Funding**

The work was supported by the Natural Science Foundation of Jiangsu Province (BK20140370), the National Science and Technology Key Projects during the Twelfth Five-Year Plan Period of China (2012ZX09401-014, 2012ZX09502001-001, 2012CB822100). A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, the Program for Changjiang Scholar and Innovative Research Team in University (IRT1075) and Jiangsu Provincial Innovative Innovative Research Team.

**Acknowledgments**

We thank the Affiliated Hospital of Jiangsu University for providing samples.

**Conflict of interest statement**

None declared.

**Abbreviations**

ACN, acetonitrile; AUC, area under the curve; CA125, cancer antigen 125; CA15-3, cancer antigen 15-3; CEA, carcinoembryonic antigen; DMSO, dimethyl sulphoxide; DTT, dithiothreitol; Fuc, fucose; Gal, galactose; LTQ-ESI-MS, linear ion-trap quadrupole-electrospray ionization mass spectrometry; MS, mass spectrometry; NaBH4, sodium borohydride; NH4HCO3, ammonium bicarbonate; PGC, porous graphitized carbon; PLS-DA, partial least squares discriminant analysis; PNGase F, peptide-N-glycosidase F; ROC, receiver-operating characteristic; TFA, trifluoroacetic acid.

**References**


Elevated level of serum glycoprotein bifucosylation and prognostic value


