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Identification of candidate biomarkers with cancer-specific glycosylation in the tissue and serum of endometrioid ovarian cancer patients by glycoproteomic analysis

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Abstract

Epithelial ovarian cancer is diagnosed less than 25% of the time when the cancer is confined to the ovary, leading to 5-year survival rates of less than 30%. Therefore, there is an urgent need for early diagnostics for ovarian cancer. Our study using glycotranscriptome comparative analysis of endometrioid ovarian cancer tissue and normal ovarian tissue led to the identification of distinct differences in the transcripts of a restricted set of glycosyltransferases involved in N-linked glycosylation. Utilizing lectins that bind to glycan structures predicted to show changes, we observed differences in lectin-bound glycoproteins consistent with some of the transcript differences. In this study, we have extended our observations by the use of selected lectins to perform a targeted glycoproteomic analysis of ovarian cancer and normal ovarian tissues. Our results have identified several glycoproteins that display tumor-specific glycosylation changes. We have verified these glycosylation changes on glycoproteins from tissue using immunoprecipitation followed by lectin blot detection. The glycoproteins that were verified were then analyzed further using existing microarray data obtained from benign ovarian adenomas, borderline ovarian adenocarcinomas, and malignant ovarian adenocarcinomas. The verified glycoproteins found to be expressed above control levels in the microarray data sets were then screened for tumor-specific glycan modifications in serum from ovarian cancer patients. Results obtained from two of these glycoprotein markers, periostin and thrombospondin, have confirmed that tumor-specific glycan changes can be used to distinguish ovarian cancer patient serum from normal serum.

Keywords

Biomarker; Glycan; Glycoprotein; Glycoproteomics; Ovarian cancer

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1 Introduction

Epithelial ovarian cancer (EOC) is the deadliest reproductive tract malignancy of women in Western countries [1]. Approximately 22 000 new cases are diagnosed each year and about 45% of these women will be alive at 5 years [2]. Methods useful for the early diagnosis of ovarian cancer could significantly improve survival rates. For example, ovarian cancer survival rates climb to greater than 90% for women diagnosed when the disease is confined to the ovary [2]. This study focuses on a specific type of ovarian cancer found to comprise 16–25% of ovarian cancer cases known as endometrioid ovarian cancer [3]. This cancer arises from the outer epithelial lining of the ovary similar to other types of ovarian cancer such as serous adenocarcinoma of the ovary, clear cell carcinoma, and mucinous carcinoma. Many endometrioid ovarian cancers are diagnosed at an earlier stage, enabling the study of early malignant lesions.

Glycosyltransferase expression levels have been shown to change in certain tumors [4–6]. Glycan structures that are added by these glycosyltransferases can be detected by specific lectins (Fig. 1A). In an earlier study, we used human endometrioid ovarian tissue, as well as a mouse model of human endometrioid ovarian cancer, and a quantitative real-time PCR approach to measure quantitative changes in the expression levels of a set of enzymes in the N-linked biosynthetic pathway [7]. We were able to identify glycosyltransferases within the N-linked pathway that had significantly increased transcript levels in the tumor tissues compared with normal. The use of lectins to fractionate complex biological samples such as tissue and serum for protein identification by MS is becoming a sensitive method to isolate potential disease markers [8–12]. Our approach targeting specific glycan structures that are changing in correlation with malignant disease has been used successfully for breast cancer [9]. In this study, we have extended this technique for normal and malignant human ovarian tissue using fractionation with multiple lectins. Our MS results have identified tumor-specific glycosylation changes on glycoprotein markers, not previously identified for ovarian cancer. In addition, we have validated these glycan changes on glycoproteins in tissue and serum collected from the patients for this study. Our approach using tissue as the initial source for glycoproteomic analysis, followed by validation in serum, has enabled us to find novel tumor-specific glycoprotein markers that may be useful for the early diagnosis of ovarian cancer.

2 Materials and methods

2.1 Tumor samples and sample preparation

Human endometrioid ovarian cancers ($n = 5$) and nondiseased human ovary tissue ($n = 4$) were obtained from women as frozen tissue from the Ovarian Cancer Institute (Atlanta, GA). Institutional Review Board approval was obtained for this research from The Georgia Institute of Technology, The University of Georgia, and Northside Hospital (Atlanta, GA). Our analysis included frozen tissue (minimum of 50 mg) wet weight that we were able to obtain from five tumor and four nondiseased age-matched ovary samples. Frozen tissue was made into a fine powder in the presence of liquid nitrogen using a mortar and pestle. Tissue powder was delipidated using a mixture of chloroform/methanol/water (4:8:3, v/v/v) as described previously [13]. Delipidated protein pellets were given an additional wash with

acetone and water (4:1) on ice for 15 min before drying under nitrogen. Delipidated pellets were stored at −80°C until use.

2.2 Lectin binding and MS sample processing

Intact proteins were extracted from the delipidated tissues using a mild detergent solution as follows: 20 mg of delipidated protein powder was dissolved in 300 μL of 50 mM Tris-Cl pH 7.5, 0.1% NP-40, 150 mM NaCl, 0.4 mM EDTA, one protease inhibitor tablet, the sample was sonicated three times for 10 s pulses at setting 5 (Vertis Virsonic microtip). The supernatant was taken after centrifugation at 10 000 rpm at 4°C for 10 min. The protein concentration of the sample was determined by BCA assay, and 600 μg of total protein lysate was dialyzed overnight at 4°C into 40 mM ammonium bicarbonate using a 4000 MWCO tube-O-dialyzer (GBiosciences). Minimal loss of protein occurred following dialysis due to the use of neutral nonbinding membrane, ≤5%. The sample was adjusted to 150 mM NaCl, 5 mM $CaCl_2$, and 5 mM $MgCl_2$ before the addition of the following lectins: biotinylated E-PHA (*Phaseolus vulgaris erythroagglutinin*), biotinylated AAL (*Aleuria aurantia*), and biotinylated DSL (*Datura stramonium*) (Vector Labs, Burlingame, CA) (10 μg each) were added and the sample was rotated at 4°C overnight. Bound lectin-reactive proteins were captured using 100 μL paramagnetic streptavidin particles (Promega) at 4°C for 2 h. After washing in 1 × PBS, captured proteins were eluted with 200 μL of 2 M urea/4 mM DTT/40 mM ammonium bicarbonate at 52°C for 1 h. The eluted fraction was separated from the paramagnetic streptavidin particles using a magnetic stand. Eluted proteins were carboxyamidomethylated by adding an equal volume of iodoacetamide (10 mg/mL in 40 mM ammonium bicarbonate) in the dark for 45 min and digested with 5 μg of sequencing grade trypsin (1:50, Promega) at 37°C overnight. Tryptic peptides were acidified with 200 μL of 1% TFA and desalting was performed using C18 spin columns (Vydac Silica C18, The Nest Group). Eluted peptides were dried in the speed vac and resuspended in 19.5 μL of buffer A (0.1% formic acid) and 0.5 μL of buffer B (80% ACN/0.1% formic acid) and filtered through a 0.2 μm filter (nanosep, PALL). Samples were loaded off-line onto a nanospray column/emitter (75 μm ×13.5 cm, New Objective) self-packed with C18 reverse-phase resin in a nitrogen pressure bomb for 10 min. Peptides were eluted *via* a 160 min linear gradient of increasing B at a flow rate of approximately 200 nL/min directly into a linear ion trap mass spectrometer (Thermo, San Jose, CA, equipped with a nanoelectrospray ion source). The top eight ions from the full MS (300–2000 *m/z*) were selected for CID fragmentation at 36% with a dynamic exclusion of two repeated counts using an exclusion time of 30 s.

2.3 Proteomic data analysis

The raw MS data were converted to mzXML using ReAdW, a software written at the Institute for Systems Biology in Seattle, WA (http://www.systemsbiology.org). MS/MS spectra were searched against the International Protein Index (IPI) human sequence database (IPI.HUMAN.v.3.26) using MyriMatch [14]. The MyriMatch search criteria included only tryptic peptides, all cysteines were presumed carboxyamidomethylated, and methionines were allowed to be oxidized. MyriMatch searches allowed a precursor error of up to 1.25 *m/z* and a fragment ion limit within 0.5 *m/z*. All ambiguous identifications that matched to multiple peptide sequences were excluded. The identified proteins (two+peptides required)

from each individual tumor and normal sample were filtered and grouped using IDPicker software [15]. IDPicker software incorporates searches against a separate reverse database, probability match obtained from MyriMatch and DeltCN scores. Information about IDPicker tools can be found at http://www.mc.vanderbilt.edu/msrc/bioinformatics/. Variance for sample processing between normal and tumor samples was calculated by measuring the number of peptides identified for proteins that adhere to the lectins in a nonglycan-dependent manner, such as serum albumin. Our results indicate 14.6%±0.16 variance between normal and tumor cases analyzed.

2.4 Western blot tissue validation

Tissue (50 mg frozen) samples were lysed in RIPA buffer (1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a mini complete protease inhibitor tablet (Roche, Indianapolis, IN) using a polytron at setting 3 for 1 min. The lysate was cleared by centrifugation at 10 000 × *g* for 10 min. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL). Lysate (500 μg) was precleared using protein A/G beads and normal IgG from the species of the primary antibody prior to immunoprecipitation. Antibodies to indicated biomarkers (2 μg) were added to 500 μg precleared lysate at 4°C for 2 h. Protein A/G beads (50 μL) were added to separate antibody-bound and -unbound proteins. Proteins bound to protein A/G were separated on 4–12% NuPage Bis Tris gels and transferred to PVDF membrane at 25 V for 1.5 h. Membranes were blocked overnight in 3% BSA/TBST buffer before lectin blot detection using a 1:5000 dilution of the following biotinylated lectins: *P. vulgaris erythroagglutinin* (E-PHA), *A. aurantia* (AAL), and *D. stramonium* (DSL), Vector Labs). Bound lectin was detected using a 1:5000 dilution of streptavidin-HRP (Vector Labs) before washing and detection using Western Lightening Plus (Perkin Elmer).

2.5 Serum validation

Serum (5 μL) was diluted in a 300 μL volume of 50 mM Tris-Cl pH 7.5, 0.1% NP-40, 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM $CaCl_2$. Biotinylated lectins E-PHA, AAL, or DSL 10 μg were added and the reactions were incubated at 4°C for 2 h. Lectin-reactive proteins were captured using 100 μL paramagnetic streptavidin particles (Promega) at 4°C for 2 h. Proteins were separated on 4–12% NuPage Bis Tris gels and transferred to PVDF membrane at 25 V for 1.5 h. Membranes were blocked overnight in 5% nonfat milk before the detection of specific proteins using the indicated antibodies.

3 Results

3.1 Multilectin glycoproteomic analysis

Lectins recognizing specific glycan structures within the N-linked glycosylation pathway (Fig. 1A) were chosen for glycoproteomic analysis based on our results demonstrating that the mRNA levels of the enzymes that synthesize these glycans were elevated 2- to 18-fold in ovarian cancer tissue relative to normal ovary [7]. The enzymes showing the largest elevations in mRNA levels were MGAT 4a, MGAT4b, MGAT5, MGAT3, and FUT8. As shown circled in Fig. 1A, the lectin *D. stramonium* (DSL) can recognize the β(1,4) branched *N*-acetylglucosamine (GlcNAc) added by the glycosyltransferases MGAT4a and MGAT4b,

as well as the β(1,6) branched *N*-acetylglucosamine added by the glyco-syltransferase MGAT5 [16]. The lectin *A. aurantia* (AAL) has a high affinity for the core α(1,6) fucose-linked product that results from the activity of the FUT8 glycosyltransferase [17, 18]. *P. vulgaris erythroagglutinin* (E-PHA) binds with the bisecting *N*-acetylglucosamine that is produced by the activity of the glycosyltransferase known as MGAT3.

Intact glycoproteins were extracted from ovarian tissue (Fig. 1A) before isolating lectin-reactive proteins using the lectins DSL, AAL, and E-PHA. The cases of ovarian cancer chosen for analysis are shown in Table 1: five cases of endometrioid ovarian cancer (two early stage and three later stage) and four cases of normal ovary tissue (age matched with tumor cases). Following multiple lectin enrichment, eluted intact glycoproteins were processed to tryptic peptides prior to MS/MS analysis. MS/MS data for each tissue sample were analyzed using the flow diagram as outlined in Fig. 1B. Myrimatch searches were used to filter *m*/*z* data against the reverse human IPI database to achieve a false discovery rate of less than 2% for proteins identified with a minimum of two peptides. We identified cumulatively 504 unique proteins from the ovarian tumor tissue and 315 unique proteins from normal ovarian tissue after multilectin enrichment. As shown in Fig. 2, more than 60% of the unique protein identifications were made with three or more peptides. Figure 2 also indicates that there was a 38% increase in the number of proteins enriched by lectins from tumor tissues relative to normal ovarian tissues. These results are expected due to the increased expression of the glycosyltransferases that add these glycan structures in ovarian cancer tissue versus nondiseased ovary [7]. The proteins identified in tumor and nondiseased cases were then grouped using the IdPicker software. Proteins that were enriched in tumor cases at a spectral abundance of 1.5 × (150%) above normal were selected. In addition, a second criterion that was applied required the protein to be present at a 1.5 × increase in at least three of the five tumor cases analyzed. Table 2 lists the proteins that remained following both data filtering criteria. Approximately, 40% of the proteins in this list contain a signal sequence and are predicted to be glycoproteins. Since we do not elute proteins from the lectin column with sugar haptens, many of the proteins not predicted to be glycoproteins by sequence analysis may be associating with glycoproteins that have bound to the lectins.

The database for annotation, visualization, and integrated discovery) was used to annotate the function of the proteins summarized in Table 2. The top three functional classifications of the proteins enriched by multilectin affinity chromatography of ovarian tumor tissue relative to nondiseased ovarian tissue were antioxidant activity (3.2E-8), glucose metabolism (8.7E-7), and cellular adhesion (9.8E-3). Considering only glycoproteins containing signal sequences, the functional categories are dominated by the anti-oxidant (*e.g.* CP, LDHA, LTF, SERPINH1, CFB, and LAMP1 (lysosomal-associated membrane protein-1)) and cellular adhesion (*e.g.* periostin (POSTN), thrombospondin 1 (THBS1), FIBLN5, MUC5b, and HSPG2) categories. Glycoproteins in these functional categories play significant roles in the progression and metastatic spread of ovarian cancer. Therefore, strategies to cotarget the glycan moieties on these glycoproteins as well as their peptide epitopes may contribute to new therapeutic strategies with greater specificities that could be useful to inhibit ovarian tumor cell adhesion and spread in the peritoneal cavity.

Most of the glycoproteins listed in Table 2 have never been exploited as potential biomarkers for ovarian cancer. The two proteins for which we are presenting further development, POSTN and THBS1, have been cited as being present in ovarian cancer [19, 20]. These proteins have not, however, been developed into potential diagnostic assays, likely due to their presence in the serum of normal patients. Our identification of tumor-specific glycosylation changes on POSTN and THBS1 for ovarian cancer tissue and patient serum appears to be novel. By extension, therefore, it is likely that other glycoproteins in Table 2 may be useful as candidate ovarian cancer markers based on glycosylation differences.

3.2 Validation studies

Glycoproteins from Table 2 were chosen for validation studies based on their reported identification in serum [21]. Since the glycoproteomic studies were performed with three lectins simultaneously, the glycoproteins markers listed in Table 2 likely have multiple, distinct glycan structures. A direct method to confirm and characterize glycan changes on glycoproteins is to immunoprecipitate the protein using an antibody directed against its polypeptide, followed by SDS-PAGE and Western blotting, then detection of the glycan of interest using a labeled lectin. In an effort to find markers for early stage disease, a single stage I case (sample 3, Table 1) was chosen for validation along with a nondiseased case (sample 7, Table 1). An example of validation is shown for the marker POSTN in Fig. 3A. Our results indicated that POSTN was reactive with the lectins AAL (panel 2, Fig 3A) and E-PHA (panel 3, Fig. 3A) only in the tumor tissue. There was no reactivity of POSTN with the lectin DSL (panel 1, Fig 3A). The tumor-selective reactivity of POSTN with AAL and E-PHA suggests that this marker would be a good candidate for serum validation.

By contrast to POSTN, the glycoprotein LAMP-1 has elevated reactivity with DSL and AAL (panels 1 and 2, Fig. 3B) in tumor tissue relative to normal tissue but very little change in E-PHA reactivity (panel 3, Fig. 3B). Equivalent levels of protein were present in each immunoprecipitation for normal and tumor tissue, evidenced by the detection of a streptavidin-reactive protein present on each blot, which was observed without lectin present. These results confirm the ability of the lectins to recognize and bind to specific glycan structures and suggest that core fucosylation (AAL reactivity, panel 2) is the most significantly changing glycan structure on LAMP-1 in ovarian cancer tissue. Although we did not detect LAMP-1 in normal tissue by proteomic analysis (Table 2), the detection of LAMP-1 lectin reactivity in normal tissue (panels 1 and 2, Fig. 3B) by immunoprecipitation and Western blot suggest that this marker is not a prime candidate for further serum validation. A technical difference in the conditions that produced the MS/MS data in Table 2 and the Western blot data shown in Fig. 3 for LAMP-1 was the denaturation of the protein prior to lectin blot analysis and not prior to the lectin chromatography/MS analysis. LAMP-1 is a heavily *N*-glycosylated protein, at least 17 potential sites of glycosylation, and it is quite possible that denaturation could render glycosylation sites accessible for lectin binding that are not exposed under the native conditions used for the multilectin affinity prior to MS/MS analysis.

During our studies, we also observed that when glycoproteins were highly reactive with E-PHA, there was low DSL reactivity; conversely, if the protein was highly positive for DSL

reactivity, the E-PHA reactivity was low (compare DSL data in Fig. 3). These results suggest that the presence of a bisecting *N*-acetylglucosamine (detected by E-PHA) could either inhibit DSL lectin binding or that the presence of the bisecting glycan inhibited glycosylation reactions on the protein to which DSL binds. The marker POSTN has been shown to express elevated β(1,6) branched N-linked glycans in invasive ductal breast carcinoma [9]. Our observation that E-PHA and DSL coreactivity does not appear to exist in a single glycoprotein population suggests that the E-PHA-reactive form of POSTN may be a selective marker for ovarian cancer. Our results suggest, therefore, that it is possible for a single glycoprotein, POSTN, to be a marker for two different types of cancer depending on specific differences in the glycans that it expresses.

Our ultimate goal is to identify glycoprotein markers that can be used to detect early stage ovarian cancers. Ovarian cancers originate *via* diverse oncogenic signaling mechanisms [22]; therefore, potential markers identified by any study may not be present at detectable levels in serum from patients with a wide variety of histological types of ovarian tumors. In order to identify markers for serum validation studies that could have the broadest applicability, we consulted the extensive microarray data set generated by The Ovarian Cancer Institute laboratory at The Georgia Institute of Technology. Microarray transcript expression profiling has been shown to discriminate benign and malignant ovarian tumors [23]. Microarray expression data were analyzed for those glycoproteins in Table 2 whose sequences predicted that they could be secreted into serum. As shown in Fig. 4A, microarray expression data revealed that the majority of potential serum glycoprotein markers identified have increased mRNA expression levels in tumors above normal. Considering the microarray data, tissue verification data, and high abundance predicted by MS spectral count analysis in ovarian cancer cases, we chose POSTN and THBS1 for initial serum validation. To validate glycan changes on glycoproteins from serum, biotinylated lectins were coupled to magnetic streptavidin beads to isolate lectin-reactive proteins prior to Western blot detection using antibodies directed against specific proteins. The validation of two candidate markers in sera is presented: POSTN (twofold increase in expression arrays) and THBS1 (fourfold increase in expression arrays) (Fig. 4A, boxed). The glycoprotein POSTN displays increased bisecting *N*-acetylglucosamine glycosylation and core fucosylation in ovarian tumor tissue, as evidenced by increased E-PHA and AAL reactivity, respectively (Fig. 3A panels 2 and 3). As shown in Fig. 4B, serum samples from four of five tumor cases have E-PHA-reactive POSTN detected above normal serum levels (Fig. 4B, panel 1, cases 1,3,4,5). Initial validation results suggest, therefore, that expression of the bisecting N-linked structure on POSTN in serum is associated with ovarian cancer. Lower grade tumors such as those in cases 3 and 4 are positive as well as higher grade tumors. Although only one band migrating at approximately 98 kDa was observed in tissue (Fig. 3A panels 2 and 3), we observed three bands migrating at approximately 98, 80, and 65 kDa in serum. The presence of smaller forms may also be due to proteolytic cleavage in serum. There is one potential N-linked glycosylation site, and it is located in the C-terminus of POSTN. The reactivity of these smaller forms of POSTN with the lectin E-PHA indicates that N-terminal cleavage of POSTN may occur after release into serum. We observed that core fucosylated (AAL reactive) POSTN was present at variable levels in serum, and its presence in nondiseased and tumor serum showed no association with the presence of malignant disease (data not

shown). These results suggest that POSTN with AAL reactivity is released into the serum from a tissue other than ovary.

The candidate marker THBS1 shows increased core fucosylation in ovarian cancer tissue relative to normal, based on AAL reactivity (data not shown). AAL precipitation and antibody analysis of THBS1in serum samples indicates that in four of five tumor cases (cases 1–4), THBS1 was more reactive with AAL compared with nondiseased serum cases (Fig. 4B, panel 2). Again, both lower grade and higher grade cases are positive for AAL reactivity with THBS1. We observed only one form of THBS1 from tissue migrating at approximately 135–140 kDa (data not shown). However, in serum, we observed a form migrating at a slightly lower molecular weight~125 kDa, as shown in Fig. 4B. The higher molecular weight glycoform can be detected at a lower level of expression in some cases, such as cases 1 and 2. THBS1 has three–four N-linked sequons, with two-three located toward the N-terminus region and one in the C-terminus region. The cleavage of THBS1 that may be occurring in serum does not affect its tumor-specific AAL reactivity, however. In these experiments, serum input amounts and quality were assessed by measuring the levels of E-PHA-reactive α-1 acid glycoprotein (Fig. 4B, panel 3). Taken together, these results identify E-PHA-reactive POSTN and AAL-reactive THBS1 as candidate markers useful in the distinguishing of sera from endometrioid ovarian cancer patients and sera from nondiseased controls. Combining the Western blot results for both markers (Fig. 4C), all five tumor cases (samples 1–5) are more lectin reactive when compared with normal serum (cases 6–9). Therefore, the cumulative detection of both glycoproteins with their tumor-specific glycan structures can distinguish the serum of ovarian endometrioid cancer patients from normal serum in 5/5 cases tested.

4 Discussion

We have used a focused approach targeting N-linked glycan structures that appear to be increased in endometrioid ovarian cancer tissue relative to normal ovary to identify potential glycoprotein markers for this cancer. This strategy has led to the identification of 47 potential tumor-specific lectin-reactive markers. We have presented tissue and serum validation methods that add confidence that in many cases the tumor-specific glycoform detected in serum has originated from the tumor. The goal of future investigations is to develop a multiglycoprotein-marker panel that when assayed together could provide an effective means for the early detection, prognosis, and monitoring of all types of ovarian cancer.

4.1 Bisecting glycans and core fucosylation in ovarian cancer

The role of bisecting glycans in most epithelial cancers is thought to suppress metastasis by suppressing the addition of branched complex N-linked glycans that can promote tumor cell migration [24–26]. EOC is a unique type of epithelial cancer due to its origination from the outer epithelial surface of the ovary that exhibits both epithelial and mesenchymal characteristics [27, 28]. Cell–cell adhesive contacts are important in the regulation of cell signaling events that promote tumorigenesis. Most epithelial tumors have a loss of cell–cell adhesion due to decreased expression of E-cadherin. However, EOC maintain E-cadherin

expression and cell–cell adhesion junctions during tumor development and progression [29]. This observation leads to the hypothesis that a possible feedback loop connecting the expression of bisecting N-linked glycans added by GnT-III and E-cadherin expression in EOC. GnT-III expression is regulated by E-cadherin-mediated cell–cell adhesion in epithelial cells [30]. Conversely, GnT-III can glycosylate E-cadherin, increasing the cell surface levels of E-cadherin and further stimulating GnT-III expression and activity[31]. The effect of increased E-cadherin on the cell surface would elevate the AKT/PI3K pathway promoting ovarian cancer cell growth and tumorigenesis [29]. Another way that bisecting glycans could promote EOC tumorigenesis is through the inhibition of apoptosis. GnT-III overexpression in HeLa cells has been shown to suppress peroxide-induced apoptosis [32]. Therefore, ovarian cancer cells under oxidative stress may activate apoptotic pathways that are then suppressed by GnT-III activity and the presence of bisecting glycans on cell surface receptors. The mechanisms of how GnT-III activity can suppress apoptosis are not fully understood, and specific acceptors of glycosylation responsible have not been identified, however. Future glycoproteomic studies on membrane glycoproteins isolated from ovarian tumor tissue could lead to the identification of cell surface glycoprotein receptors that may be potential therapeutic targets.

Core fucosylation, a common N-linked glycan modification, is increased in and serves as a marker for several cancers [33–37]. Increased core fucosylation on glycoproteins promotes growth factor signaling, since FUT8−/− mice are small and die during prenatal development [38]. FUT8 expression has also been implicated in the regulation of EGFR and PDGF receptor internalization and signaling [39]. Core fucosylation has also been linked to enhanced E-cadherin-mediated cell–cell adhesion in colon cancer cells by reducing the turnover of cell surface E-cadherin [40]. Therefore, the cumulative effect of increased bisecting *N*-acetylglucosamine and core fucosylation on E-cadherin would likely promote ovarian cancer tumorigenesis.

4.2 Cell stress and glycosylation changes

Our glycoproteomic data has identified several non-N-linked-glycosylated proteins enriched by multilectin chromatography of ovarian tumor extracts. Many of these proteins are HSP and chaperones (GRP78, HSPB1, YWHAG, and CCT5), protein disulfide isomerases (PDIA3, PDIA4, and PDIA6), and glucose metabolism enzymes (GPI, PGK1, PGD, and PGAM1). Tumor cells that are proliferating rapidly create a glucose-deprived, hypoxic environment [41]. The classes of non-N-linked-glycosylated proteins observed to be enriched by multilectin chromatography may be managing this type of cellular stress. Many of these proteins such as GRP78 have been identified on the cell surface in previous proteomic studies [42]. GRP78 is induced by glucose deprivation and acts as a cell survival factor capable of inhibiting apoptosis in many tumors [43, 44]. While GRP78 may not be an ideal biomarker for the diagnosis of ovarian cancer due to its increased levels in many different types of cancer, it may, however, be useful in directing therapeutics [45]. Although GRP78 contains no predicted N-linked glycosylation sequons, we sought to test whether GRP78 isolated by immunoprecipitation would be bound directly by lectins. We immunoprecipitated GRP78 from ovarian cancer tissues, subjected the bound proteins to SDS-PAGE and Western blotting, but observed no reactivity with the lectins used in this

study (data not shown). Therefore, GRP78 must be binding tightly enough to glycoproteins to be enriched by lectin affinity. Which glycoproteins GRP78 is binding with on the surface of ovarian cancer cells is currently unknown. Future studies identifying the cell surface glycoproteins GRP78 may be binding with may provide targets for peptide therapeutic strategies to block the antiapoptotic activity of GRP78. Our approach solubilizing proteins in mild detergent would not allow for the identification of possible membrane glycoproteins that may be GRP78 cell surface-binding partners.

4.3 Glycoprotein markers and potential involvement in ovarian cancer spread

Ovarian cancer cells typically form multicellular aggregates and may spread by attachment to the peritoneal abdominal wall lining [46]. This type of nonhematological tumor spread may be influenced by the extracellular matrix proteins identified in this study, such as POSTN, BGN, HSPG2, THBS1, and FIBLN5, and corresponding interactions with their cell receptors. A recent proteomic study to identify expression profiles associated with invasive potential in ovarian cancer cell lines found that the most significantly enriched signaling pathway promoting invasivity was extracellular matrix receptor signaling [47]. The adhesive glycoproteins that we have validated as glycomarkers for ovarian cancer, POSTN and THBS1, have been implicated in promoting tumor spread [19, 20]. Future studies will focus on determining if increased core fucosylation and bisecting N-linked glycans on these glycoproteins augment ovarian cancer peritoneal adhesion with a focus on identifying potential mechanisms that could be targeted to block EOC peritoneal adhesion.

4.4 Relationship of the results of this study to CA-125, a glycoprotein marker for ovarian cancer

The best-known glycoprotein marker for ovarian cancer is CA-125 (MUC16). Serum protein levels of this marker may be used to monitor ovarian cancer patients during treatment; however, due to variable concentrations of CA-125 in benign diseases, this assay has not been in routine as a diagnostic assay [48]. CA-125 is a large mucin protein containing both N- and O-linked glycans, and its complexity has hampered detailed analysis of its glycan structures. However, the glycosylation patterns of CA-125 and certain acute-phase glycoproteins have been documented to change in ovarian cancer [49–51]. For example, Jankovic *et al.* compared the glycans of CA-125 isolated from amniotic fluid to CA-125 from the OVCAR3 ovarian cancer cell line and found a significant increase in the reactivity of OVCAR3 CA-125 with the lectin E-PHA compared with CA-125 from amniotic fluid [51]. These data agree with our finding that bisecting N-linked glycans are elevated in ovarian cancer tissue relative to normal ovary [7]. Despite these findings, however, we were unable to detect CA-125 in our glycoproteomic analysis of endometrioid ovarian cancer tissue, which may reflect differences between endometrioid ovarian cancer and other adenocarcinomas of the ovary. We did identify another mucin, MUC5b, that showed increased binding to the lectins used in this study, suggesting that different types of ovarian cancers may secrete different dominate mucins. These data suggest that assays targeting mucin glycoproteins may not have sufficient sensitivity for a variety of histological types and grades of ovarian tumors. The ability to correlate focused glycoproteomic data with genomic microarray data from a diverse sampling of histological type ovarian tumors will

likely lead to glycomarkers with increased sensitivity for many histological subtypes of ovarian cancer.

In conclusion, the glycoproteomic results presented offers initial validation that glycoproteins with tumor-specific glycan changes can be used to distinguish malignant ovarian tissue and serum from normal ovarian tissue and serum. The glycosylated candidate markers and nonglycosylated candidate markers identified with tumor-specific lectin affinity are promising for the detection and potential therapeutic intervention of endometrioid ovarian cancer and possibly other forms of ovarian cancer.

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Abbreviations

EOC	epithelial ovarian cancer
IPI	International Protein Index
POSTN	periostin
THBS1	thrombospondin 1

References

1. Ozols RF, Bookman MA, Connolly DC, Daly MB, et al. Focus on epithelial ovarian cancer. Cancer Cell. 2004; 5:19–24. [PubMed: 14749123]
2. Hayat MJ, Howlader N, Reichman ME, Edwards BK. Cancer statistics, trends, and multiple primary cancer analyses from the Surveillance, Epidemiology, and End Results (SEER) Program. Oncologist. 2007; 12:20–37. [PubMed: 17227898]
3. Storey DJ, Rush R, Stewart M, Rye T, et al. Endometrioid epithelial ovarian cancer: 20 years of prospectively collected data from a single center. Cancer. 2008; 112:2211–2220. [PubMed: 18344211]
4. Buckhaults P, Chen L, Fregien N, Pierce M. Transcriptional regulation of N-acetylglucosaminyltransferase V by the src oncogene. J Biol Chem. 1997; 272:19575–19581. [PubMed: 9235963]
5. Seales EC, Jurado GA, Singhal A, Bellis SL. Ras oncogene directs expression of a differentially sialylated, functionally altered beta1 integrin. Oncogene. 2003; 22:7137–7145. [PubMed: 14562042]
6. Takahashi T, Ikeda Y, Miyoshi E, Yaginuma Y, et al. alpha1,6 fucosyltransferase is highly and specifically expressed in human ovarian serous adenocarcinomas. Int J Cancer. 2000; 88:914–919. [PubMed: 11093814]
7. Abbott KL, Nairn AV, Hall EM, Horton MB, et al. Focused glycomic analysis of the N-linked glycan biosynthetic pathway in ovarian cancer. Proteomics. 2008; 8:3210–3220. [PubMed: 18690643]
8. Yang Z, Harris LE, Palmer-Toy DE, Hancock WS. Multilectin affinity chromatography for characterization of multiple glycoprotein biomarker candidates in serum from breast cancer patients. Clin Chem. 2006; 52:1897–1905. [PubMed: 16916992]
9. Abbott KL, Aoki K, Lim JM, Porterfield M, et al. Targeted glycoproteomic identification of biomarkers for human breast carcinoma. J Proteome Res. 2008; 7:1470–1480. [PubMed: 18271524]

10. Mechref Y, Madera M, Novotny MV. Glycoprotein enrichment through lectin affinity techniques. Methods Mol Biol. 2008; 424:373–396. [PubMed: 18369876]
11. Kim YS, Son OL, Lee JY, Kim SH, et al. Lectin precipitation using phytohemagglutinin-L(4) coupled to avidin-agarose for serological biomarker discovery in colorectal cancer. Proteomics. 2008; 8:3229–3235. [PubMed: 18633972]
12. Pierce, JM. Cancer Glycomics. In: Cummings, RD.; Pierce, JM., editors. Handbook of Glycomics. Academic Press; San Diego: 2009.
13. Aoki K, Perlman M, Lim JM, Cantu R, et al. Dynamic developmental elaboration of N-linked glycan complexity in the *Drosophila melanogaster* embryo. J Biol Chem. 2007; 282:9127–9142. [PubMed: 17264077]
14. Tabb DL, Fernando CG, Chambers MC. MyriMatch: Highly accurate tandem mass spectral peptide identification by multivariate hypergeometric analysis. J Proteome Res. 2007; 6:654–661. [PubMed: 17269722]
15. Zhang B, Chambers MC, Tabb DL. Proteomic parsimony through bipartite graph analysis improves accuracy and transparency. J Proteome Res. 2007; 6:3549–3557. [PubMed: 17676885]
16. Wu AM, Lisowska E, Duk M, Yang Z. Lectins as tools in glycoconjugate research. Glycoconj J. 2009; 26:899–913. [PubMed: 18368479]
17. Nagata Y, Fukumori F, Sakai H, Hagiwara T, et al. Crystallization and characterization of a lectin obtained from a mushroom, *Aleuria aurantia*. Biochim Biophys Acta. 1991; 1076:187–190. [PubMed: 1998719]
18. Iskratsch T, Braun A, Paschinger K, Wilson IB. Specificity analysis of lectins and antibodies using remodeled glycoproteins. Anal Biochem. 2009; 386:133–146. [PubMed: 19123999]
19. Gillan L, Matei D, Fishman DA, Gerbin CS, et al. Periostin secreted by epithelial ovarian carcinoma is a ligand for alpha(V)beta(3) and alpha(V)beta(5) integrins and promotes cell motility. Cancer Res. 2002; 62:5358–5364. [PubMed: 12235007]
20. Bignotti E, Tassi RA, Calza S, Ravaggi A, et al. Gene expression profile of ovarian serous papillary carcinomas: identification of metastasis-associated genes. Am J Obstet Gynecol. 2007; 196:245, e241–211. [PubMed: 17346539]
21. Liu T, Qian WJ, Gritsenko MA, Camp DG II, et al. Human plasma N-glycoproteome analysis by immunoaffinity subtraction, hydrazide chemistry, and mass spectrometry. J Proteome Res. 2005; 4:2070–2080. [PubMed: 16335952]
22. Aunoble B, Sanches R, Didier E, Bignon YJ. Major oncogenes and tumor suppressor genes involved in epithelial ovarian cancer (review). Int J Oncol. 2000; 16:567–576. [PubMed: 10675491]
23. Warrenfeltz S, Pavlik S, Datta S, Kraemer ET, et al. Gene expression profiling of epithelial ovarian tumours correlated with malignant potential. Mol Cancer. 2004; 3:27. [PubMed: 15471544]
24. Schachter H. Biosynthetic controls that determine the branching and microheterogeneity of protein-bound oligosaccharides. Adv Exp Med Biol. 1986; 205:53–85. [PubMed: 3538817]
25. Takahashi M, Kuroki Y, Ohtsubo K, Taniguchi N. Core fucose and bisecting GlcNAc, the direct modifiers of the N-glycan core: their functions and target proteins. Carbohydr Res. 2009; 344:1387–1390. [PubMed: 19508951]
26. Lau KS, Partridge EA, Grigorian A, Silvescu CI, et al. Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. Cell. 2007; 129:123–134. [PubMed: 17418791]
27. Lee Y, Miron A, Drapkin R, Nucci MR, et al. A candidate precursor to serous carcinoma that originates in the distal fallopian tube. J Pathol. 2007; 211:26–35. [PubMed: 17117391]
28. Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC. Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr Rev. 2001; 22:255–288. [PubMed: 11294827]
29. De Santis G, Miotti S, Mazzi M, Canevari S, Tomassetti A. E-cadherin directly contributes to PI3K/AKT activation by engaging the PI3K-p85 regulatory subunit to adherens junctions of ovarian carcinoma cells. Oncogene. 2009; 28:1206–1217. [PubMed: 19151754]
30. Akama R, Sato Y, Kariya Y, Isaji T, et al. N-acetylglucosaminyltransferase III expression is regulated by cell-cell adhesion via the E-cadherin-catenin-actin complex. Proteomics. 2008; 8:3221–3228. [PubMed: 18690644]

31. Liwosz A, Lei T, Kukuruzinska MA. N-glycosylation affects the molecular organization and stability of E-cadherin junctions. J Biol Chem. 2006; 281:23138–23149. [PubMed: 16682414]
32. Shibukawa Y, Takahashi M, Laffont I, Honke K, Taniguchi N. Down-regulation of hydrogen peroxide-induced PKC delta activation in N-acetylglucosaminyltransferase III-transfected HeLaS3 cells. J Biol Chem. 2003; 278:3197–3203. [PubMed: 12427758]
33. Comunale MA, Mehta A. Glycoproteomic analysis by two-dimensional electrophoresis. Methods Mol Biol. 2009; 520:59–74. [PubMed: 19381947]
34. Comunale MA, Wang M, Hafner J, Krakover J, et al. Identification and development of fucosylated glycoproteins as biomarkers of primary hepatocellular carcinoma. J Proteome Res. 2009; 8:595–602. [PubMed: 19099421]
35. Mehta A, Block TM. Fucosylated glycoproteins as markers of liver disease. Dis Markers. 2008; 25:259–265. [PubMed: 19126969]
36. Inamori K, Endo T, Gu J, Matsuo I, et al. N-acetyl-glucosaminyltransferase IX acts on the GlcNAc beta 1,2-Man alpha 1-Ser/Thr moiety, forming a 2,6-branched structure in brain O-mannosyl glycan. J Biol Chem. 2004; 279:2337–2340. [PubMed: 14617637]
37. Nakagawa T, Miyoshi E, Yakushijin T, Hiramatsu N, et al. Glycomic analysis of alpha-fetoprotein L3 in hepatoma cell lines and hepatocellular carcinoma patients. J Proteome Res. 2008; 7:2222–2233. [PubMed: 18479159]
38. Wang X, Gu J, Miyoshi E, Honke K, Taniguchi N. Phenotype changes of Fut8 knockout mouse: core fucosylation is crucial for the function of growth factor receptor(s). Methods Enzymol. 2006; 417:11–22. [PubMed: 17132494]
39. Wang X, Gu J, Ihara H, Miyoshi E, et al. Core fucosylation regulates epidermal growth factor receptor-mediated intracellular signaling. J Biol Chem. 2006; 281:2572–2577. [PubMed: 16316986]
40. Osumi D, Takahashi M, Miyoshi E, Yokoe S, et al. Core fucosylation of E-cadherin enhances cell-cell adhesion in human colon carcinoma WiDr cells. Cancer Sci. 2009; 100:888–895. [PubMed: 19302290]
41. Brahimi-Horn MC, Chiche J, Pouyssegur J. Hypoxia and cancer. J Mol Med. 2007; 85:1301–1307. [PubMed: 18026916]
42. Shin BK, Wang H, Yim AM, Le Naour F, et al. Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function. J Biol Chem. 2003; 278:7607–7616. [PubMed: 12493773]
43. Yeung BH, Kwan BW, He QY, Lee AS, et al. Glucose-regulated protein 78 as a novel effector of BRCA1 for inhibiting stress-induced apoptosis. Oncogene. 2008; 27:6782–6789. [PubMed: 18776923]
44. Kumar Y, Tatu U. Stress protein flux during recovery from simulated ischemia: induced heat shock protein 70 confers cytoprotection by suppressing JNK activation and inhibiting apoptotic cell death. Proteomics. 2003; 3:513–526. [PubMed: 12687618]
45. Lee AS. GRP78 induction in cancer: Therapeutic and prognostic implications. Cancer Res. 2007; 67:3496–3499. [PubMed: 17440054]
46. Burleson KM, Boente MP, Pambuccian SE, Skubitz AP. Disaggregation and invasion of ovarian carcinoma ascites spheroids. J Transl Med. 2006; 4:6. [PubMed: 16433903]
47. Sodek KL, Evangelou AI, Ignatchenko A, Agochiya M, et al. Identification of pathways associated with invasive behavior by ovarian cancer cells using multidimensional protein identification technology (MudPIT). Mol Biosyst. 2008; 4:762–773. [PubMed: 18563251]
48. Clarke-Pearson DL. Clinical practice screening for ovarian cancer. N Engl J Med. 2009; 361:170–177. [PubMed: 19587342]
49. Saldova R, Royle L, Radcliffe CM, Abd Hamid UM, et al. Ovarian cancer is associated with changes in glycosylation in both acute-phase proteins and IgG. Glycobiology. 2007; 17:1344–1356. [PubMed: 17884841]
50. Saldova R, Wormald MR, Dwek RA, Rudd PM. Glycosylation changes on serum glycoproteins in ovarian cancer may contribute to disease pathogenesis. Dis Markers. 2008; 25:219–232. [PubMed: 19126966]

51. Jankovic MM, Milutinovic BS. Glycoforms of CA125 antigen as a possible cancer marker. Cancer Biomark. 2008; 4:35–42. [PubMed: 18334732]

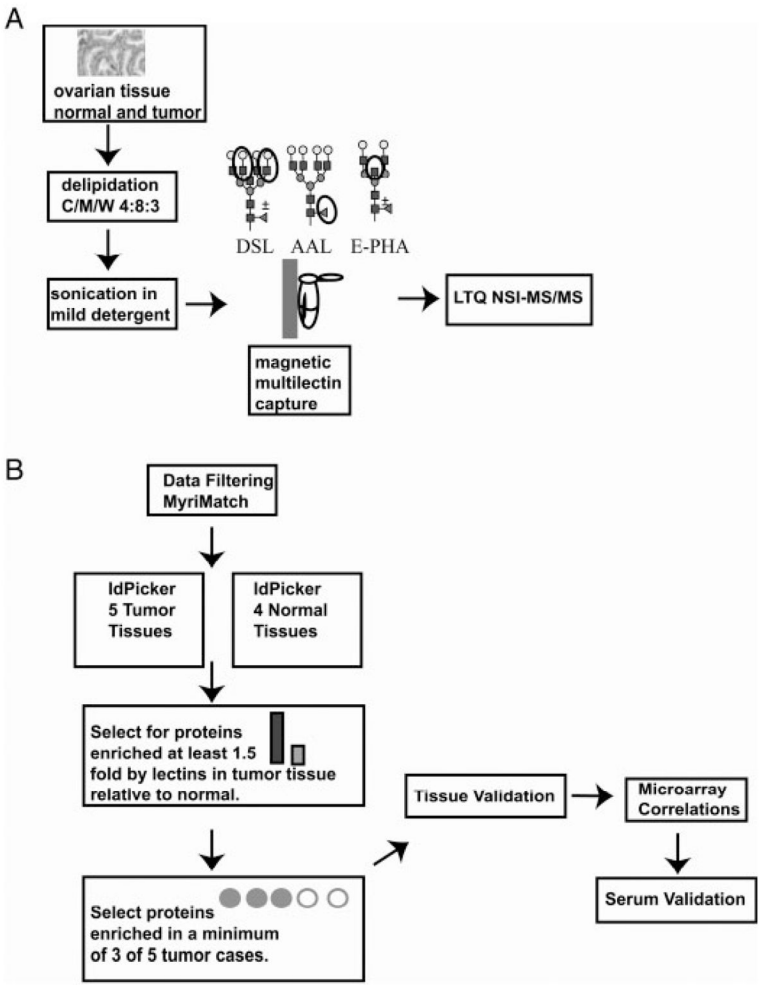

Figure 1.

(A) Schematic flow of the multi-lectin glycoproteomic method used in this study. The glycan structures targeted are circled in the structures displayed. (B) Flow diagram illustrating the data analysis and filtering methods.

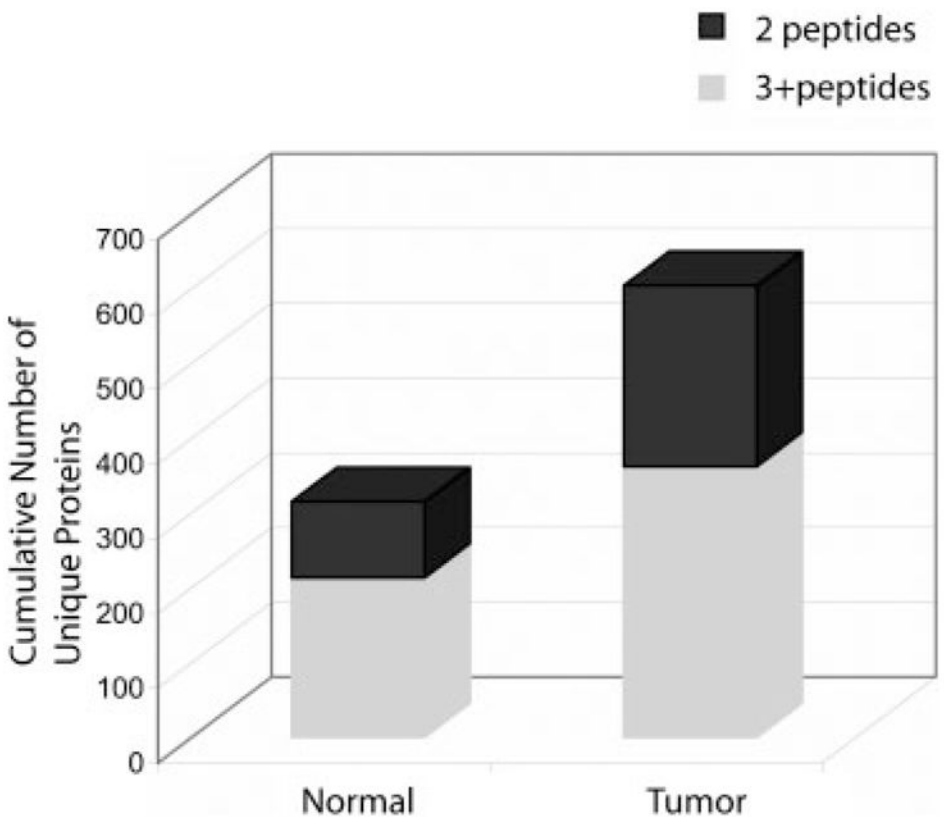

Figure 2.
Graphical presentation of cumulative proteomic data.

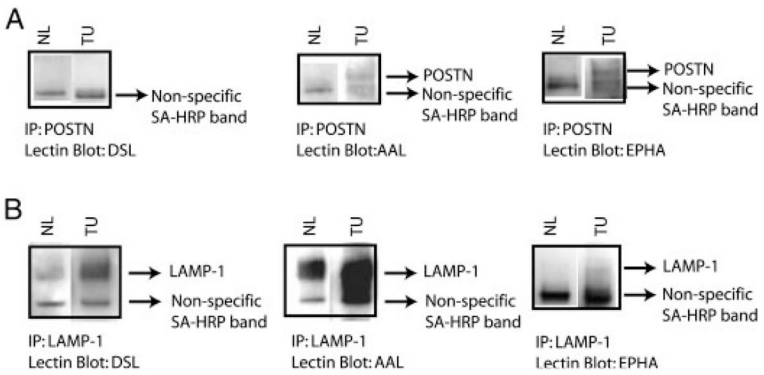

Figure 3.

Tissue validation. (A) Lectin blot analysis of POSTN immunoprecipitations. POSTN was immunoprecitated from 500 μg of total cell lysate using a polyclonal antibody (Abcam, Cambridge, MA) prior to separation on 4–12% polyacrylamide gel and transfer to PVDF membrane. Blots were probed with biotinylated lectins (1:5000) and detected using streptavidin-coupled HRP (1:5000) and chemiluminescent development. (B) Lectin blot analysis of LAMP-1 immunoprecipitation reactions. LAMP-1 was immunoprecipitated from 500 μg of total cell lysates (normal-NL and tumor-TU) using a monoclonal antibody (E-Biosciences, San Diego, CA) detected by lectin blot as described above.

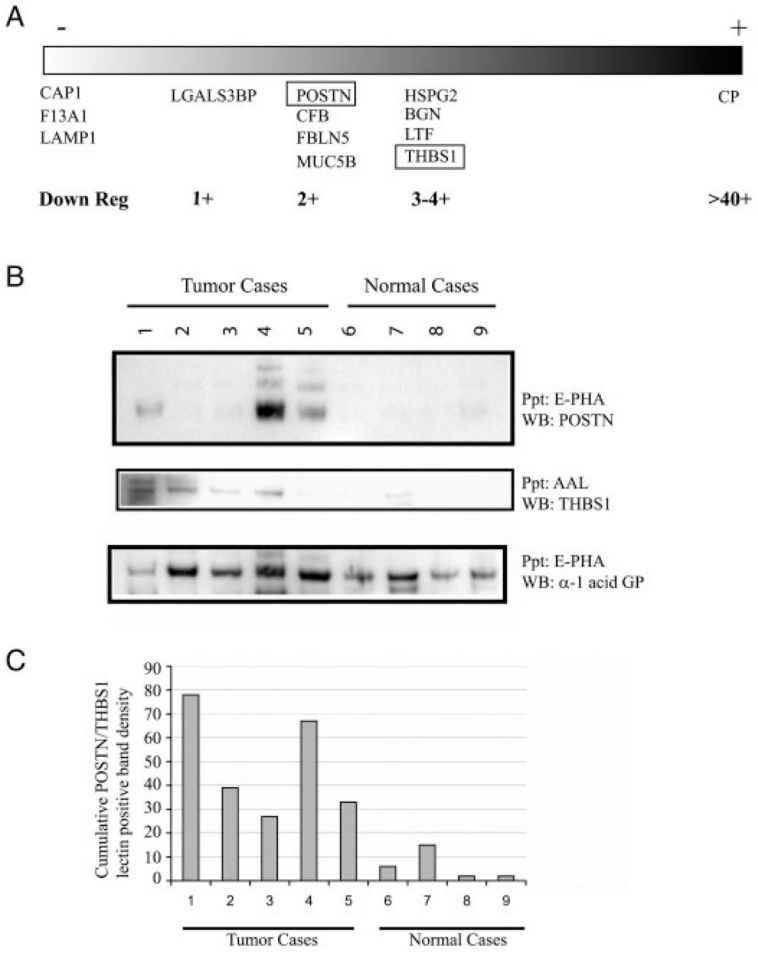

Figure 4.

Microarray data and serum validation. (A) Visualization of normalized micro-array data (performed at the Georgia Institute of Technology and The Ovarian Cancer Institute) for glycoproteins from Table 2 selected for possible serum validation. The averaged fold-increase in expression levels in tumor tissue relative to normal tissue is shown below. Gene name abbreviations and IPI accessions are provided in Table 2. (B) Western blot analysis of serum lectin precipitation reactions. The following antibodies were used: POSTN (Abcam, 1:1000), THBS1 (Santa Cruz, 1:250), and α1-acid GP (Abcam, 1:1000). (C) Cumulative Western blot data from two experiments was analyzed by densitometry using the Image J. Averaged scaled densitometry values for POSTN and THBS1 were added.

Table 1

Patient information and proteins detected following multilectin proteomics of ovarian tissue

Sample no.	Histology	Tumor stage	Tumor grade	Unique proteins
1	Endometrioid	III–IV	2	525
2	Endometrioid	IIIa	3	416
3	Endometrioid	Ia	2	306
4	Endometrioid	Ia–Ic	2	328
5	Endometrioid	IIIb–IIIc	3	416
6	Normal	n/a	n/a	242
7	Normal	n/a	n/a	258
8	Normal	n/a	n/a	158
9	Normal	n/a	n/a	213

n/a, not applicable.

Table 2

Multilectin reactive proteins with increased spectra in tumor relative to normal for at least three of five cases analyzed

IPI accession[a)]	Gene name	Molecular weight	Description	Cumulative spectral counts tumor	Cumulative spectral counts normal	Cellular location	Predicted glycoprotein	In *N*-glycoprotein plasma proteome study[b)]
IPI00003362.2	GRP78	72	Glucose-regulated protein 78	16	0	Cell surface	No	No
IPI00003881.5	HNRNPF	45	Heterogeneous nuclear ribonucleoprotein F	7	0	Cytoplasm	No	No
IPI00004503.5	LAMP1	44	Lysosomal-associated membrane protein 1	8	0	Membrane	Yes	Yes
IPI00007960.4	POSTN	93	Periostin	20	5	Extracellular region	Yes	Yes
IPI00008274.7	CAP1	51	Adenylate cyclase-associated protein 1	7	0	Membrane	Yes	Yes
IPI00009904.1	PDIA4	72	Protein disulfide isomerase family A, member 4	4	0	ER	No	No
IPI00010720.1	CCT5	57	Chaperonin containing TCP1, subunit 5 (epsilon)	8	0	Cytoplasm	No	No
IPI00010790.1	BGN	42	Biglycan precursor	34	8	Extracellular region	Yes	No
IPI00010796.1	P4HB	122	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase)	14	0	ER/golgi	No	No
IPI00017601.1	CP	57	Ceruloplasmin	8	0	Extracellular region	Yes	Yes
IPI00018219.1	TGFBI	75	Transforming growth factor, β induced	16	6	Extracellular region	No	No
IPI00019502.3	MYH9	226	Myosin, heavy chain 9, nonmuscle	34	7	Cytoplasm, membrane	No	No
IPI00019591.1	CFB	86	Complement factor B	7	0	Cell surface	Yes	Yes
IPI00020501.1	MYH11	227	Myosin, heavy chain 11, smooth muscle	21	5	Cytoplasm	No	No
IPI00020599.1	CRTC	48	Calreticulin	14	3	ER	No	No
IPI00022977.1	CKB	42	Creatine kinase-brain	7	0	Cytoplasm	No	No
IPI00023673.1	LGALS3BP	65	Lectin, galactoside-binding, soluble, 3 binding protein	8	0	Extracellular region, membrane	Yes	Yes
IPI00024284.4	HSPG2	469	Heparan sulfate proteoglycan 2	7	0	Extracellular region	Yes	Yes
IPI00025252.1	PDIA3	57	Protein disulfide isomerase family A, member 3	16	0	ER	No	No

IPI accession[a)]	Gene name	Molecular weight	Description	Cumulative spectral counts tumor	Cumulative spectral counts normal	Cellular location	Predicted glycoprotein	In *N*-glycoprotein plasma proteome study[b)]
IPI00025512.2	HSPB1	23	HSP-1 27 kDa	13	4	Cell surface	No	No
IPI00027497.5	GPI	63	Glucose-6-phosphate isomerase	7	0	ER	No	No
IPI00032140.4	SERPINH1	46	Serine protease inhibitor H1	19	8	ER/golgi	Yes	No
IPI00169383.3	PGK1	45	Phosphoglycerate kinase 1	16	3	Cytoplasm	No	No
IPI00186290.6	EEF2	93	Elongation factor 2	10	3	Cytoplasm	No	No
IPI00215914.5	ARF1	21	ADP-Ribosylation factor 1	5	0	Golgi	No	No
IPI00216049.1	HNRNPK	51	Heterogeneous nuclear ribonucleoprotein K	9	0	Cytoplasm	No	No
IPI00217966.7	LDHA	37	Lactate dehydrogenase A	18	2	Cytoplasm	Yes	No
IPI00219525.10	PGD	53	Phosphogluconate dehydrogenase	5	2	Cytoplasm	No	No
IPI00219713	FIBG	51	Fibrinogen-γ	17	2	Extracellular region	Yes	No
IPI00220301.5	PRDX6	25	Peroxiredoxin 6	5	0	Cytoplasm	No	No
IPI00220642.7	YWHAG	28	14-3-3-γ	5	0	Cytoplasm	No	No
IPI00291006.1	MDH2	35	Malate dehydrogenase 2, NAD (mitochondrial)	6	0	Mitochondrial membrane	No	No
IPI00296099.6	THBS1	129	Thrombospondin1	22	0	Extracellular region	Yes	Yes
IPI00297550.8	F13A1	83	Coagulation factor XIII, A1 polypeptide	8	0	Extracellular region	Yes	Yes
IPI00298860.5	LTF	78	Lactotransferrin	4	0	Extracellular region	Yes	No
IPI00298994.5	TLN1	269	Talin 1	9	0	Membrane	No	No
IPI00299571.5	PDIA6	54	Protein disulfide isomerase family A, member 6	15	0	ER/golgi	No	No
IPI00376005.2	EIF5A	20	Eukaryotic translation initiation factor 5A	5	0	Cytoplasm	No	No
IPI00382428.6	FBLN5	60	Fibulin 5	5	2	Extracellular region	Yes	No
IPI00426051.3	IGHG2	51	Similar to hCG2038920	8	0	Unknown	Yes	No
IPI00478003.1	A2M	163	α-2-Macroglobulin	31	2	Extracellular region	Yes	No
IPI00479217.1	HNRNPU	89	Heterogeneous nuclear ribonucleoprotein U	5	0	Cell surface	No	No
IPI00549725.6	PGAM1	29	Phosphoglycerate mutase 1 (brain)	5	0	Cytoplasm	No	No
IPI00550363.3	TAGLN2	24	Transgelin 2	13	2	Cytoplasm	No	No

IPI accession[a)]	Gene name	Molecular weight	Description	Cumulative spectral counts tumor	Cumulative spectral counts normal	Cellular location	Predicted glycoprotein	In *N*-glycoprotein plasma proteome study[b)]
IPI00759776.1	ACTN1	103	Actinin 1 isoform b	14	0	Cytoplasm	No	No
IPI00719373.1	IGL2	23	Immunoglobulin lambda locus	13	8	Extracellular region	Yes	No
IPI00787849.1	MUC5B	597	Mucin 5B	18	0	Extracellular region	Yes	No

a)IPI database.

b)Published analysis of the N-linked glycoproteins from human plasma (Liu *et al.*, 2005 [21]].