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Markers of Ovarian Cancer Using a Glycoprotein/Antibody Array

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Markers of Ovarian Cancer Using a Glycoprotein/Antibody Array

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Glycoproteins, Biomarkers, lectins, microarrays

Profiling of the glycan structures that differ between the serum of serous ovarian cancer and benign conditions have been performed using lectin arrays for biomarker studies. These arrays have used 16 different lectins which respond to specific glycan structural moieties of glycoproteins in patient serum. It has been found that there are distinct changes in the level of fucosylation between cancer and benign using LCA, AAL and UEA lectins which detect both core and outer arm glycosylation. Some difference was also observed for SNA which detects 2,6 sialylation. Using these lectins we have been able to extract the glycoproteins from patient serum and are evaluating the differentially expressed glycoproteins based on mass spec and glycoarray technologies. We have identified 10-12 glycoproteins which show distinct changes in expression between benign conditions and stage 3c serous ovarian cancer. These glycoproteins will be used for further validation studies in future work.
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Introduction: We are developing a lectin/antibody assay for biomarker discovery and validation in ovarian cancer (1-3). The novel concept here as opposed to most current marker studies is that the marker depends upon structural changes of glycosylation on a specific protein rather than on the abundance of the protein itself. Many of the markers may be relatively high abundance glycoproteins found in serum, but it is the glycan group whose structure changes in a well regulated manner during cancer progression that will be monitored rather than the abundance of the glycoprotein itself. These glycoprotein markers may be present in many cancers but will be shown to change in a very specific manner for each different type of cancer and stage of cancer and also for other benign conditions.

Description of Progress:

We have been currently working according to the SOW and most recently have focused on the following tasks. In the prior year we had described the work on our optimal performing candidates (4) and now are working on evaluating glycosylation changes of these candidates (5).

Task 2. Further evaluate sialoglycoproteins/lectin pairs as potential markers using lectin-based ELISA assays and ELISA assays (Months 18-28)

a.) Lectin was coated to 96-well plates to produce a lectin-based ELISA assay.
b.) Use an open test set of 21 early stage ovarian cancers, 29 late stage ovarian cancers, 18 ovarian benign disease, and 15 normal controls to determine from original set of 5 candidate markers the 2-3 markers with the optimal response characteristics. These are currently being further verified using a larger sample set in our work.

SNA-based ELISA Assay. SNA-based ELISA assay was used to detect the expression of a particular sialylated glycoprotein from original serum samples without depletions. The results showed that CLUS, HEMO, and CFH had significantly lower response to SNA in cancer sera than in benign diseases or healthy controls (p<0.05, Figure 1a). We also found the decreased sialylation levels of CLUS, and HEMO in early stage cancer sera (Figure 1a). A ROC curve was constructed for each of the three changed sialylated proteins to differentiate ovarian cancer from benign diseases or healthy controls (Figure 1b). The combination of the 3 proteins had an AUC of 0.873 with a specificity of 73% at a sensitivity of 91% for detecting early stage ovarian cancer (Figure 1b).

Figure 1. Sialylated protein alterations confirmed by SNA-based ELISA assay. (a) Comparison of response
intensity of CLUS, HEMO, and CFH to SNA in normal healthy controls, benign diseases, stage I/II ovarian cancer, and stage III ovarian cancer. The sialylated protein levels with significant changes between pairwise comparisons were indicated (*p<0.05, **p<0.01). (b) ROC curve for sialylated CLUS, HEMO, and CFH to distinguish ovarian cancer from benign diseases or healthy controls.

**ELISA assays.** ELISA was applied to further verify the variation of the significantly changed proteins detected by quantitative MS method and lectin-based ELISA assay, which include CLUS, LRG1, HEMO, VDB, and CFH. Among the five proteins, VDB, HEMO, and CFAB had no statistically significant difference between all pairwise comparisons (p>0.05), and LRG1, and CLUS showed significant difference for the specific pairwise comparisons (p<0.05), as shown in Figure 2a. The combination of CLUS and LRG1 (AUC=0.837) showed improved performance for distinguishing stage III ovarian cancer from benign diseases compared to CA125 alone (AUC=0.811). In differentiating early stage ovarian cancer from benign diseases or healthy controls, LRG1 showed comparable performance to CA125, as shown in Figure 2b.

![Figure 2](image.png)

**Figure 2.** Underlying protein levels of individual candidate biomarkers measured by ELISA kits. (a) The levels of LRG1 and CLUS showed significant changes between pairwise comparisons (*p<0.05, **p <0.01). (b) ROC analyses for CLUS, LRG1 and CA125 to differentiate ovarian cancer from healthy controls or benign diseases.

**Key Research Accomplishments:**

- a potential panel of 2-3 markers for distinguishing stage I/II serous cancer in serum from normals or
benign samples.
- a potential panel of 3 markers for distinguishing stage III serous cancer in serum from normal controls or benign diseases

**Reportable Outcomes:** Postdoctoral associate Jing Wu has been trained in marker studies, elisas, lectin-elisas and mass spectrometry. She has identified a potential panel of markers of ovarian cancer for further confirmation and validation.

**Publications:**

**Conclusion:**
We have applied a comprehensive strategy for identification and confirmation of glycoprotein biomarkers for detection of serous ovarian cancer. Lectin-based ELISA and ELISA were respectively used to verify the variance of these changed glycoproteins in their glycan levels and underlying core protein levels. We found sialylated CLUS, CFH and HEMO could be used to distinguish stage III cancer from benign diseases or healthy controls. Core protein levels of CLUS and LRG1 are complementary to CA125 with improved sensitivity and specificity for differentiating stage III cancer from benign. We also found LRG1, CLUS and HEMO could be used as candidate biomarkers for detection of ovarian cancer in early stage. The next step is to apply a larger cohort of patients of ovarian cancer to verify our present findings.

**References:**
Identification and Confirmation of Differentially Expressed Fucosylated Glycoproteins in the Serum of Ovarian Cancer Patients Using a Lectin Array and LC–MS/MS

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Supporting Information

ABSTRACT: In order to discover potential glycoprotein biomarkers in ovarian cancer, we applied a lectin array and Exactag labeling based quantitative glycoproteomics approach. A lectin array strategy was used to detect overall lectin-specific glycosylation changes in serum proteins from patients with ovarian cancer and those with benign conditions. Lectins, which showed significant differential response for fucosylation, were used to extract glycoproteins that had been labeled using isobaric chemical tags. The glycoproteins were then identified and quantified by LC–MS/MS, and five glycoproteins were found to be differentially expressed in the serum of ovarian cancer patients compared to benign diseases. The differentially expressed glycoproteins were further confirmed by lectin-ELISA and ELISA assay. Corticosteroid-binding globulin (CBG), serum amyloid p component (SAP), complement factor B (CFAB), and histidine-rich glycoprotein (HRG) were identified as potential markers for differentiating ovarian cancer from benign diseases or healthy controls. A combination of CBG and HRG (AUC = 0.825) showed comparable performance to CA125 (AUC = 0.829) in differentiating early stage ovarian cancer from healthy controls. The combination of CBG, SAP, and CA125 showed improved performance for distinguishing stage III ovarian cancer from benign diseases compared to CA125 alone. The ability of CBG, SAP, HRG, and CFAB to differentiate the serum of ovarian cancer patients from that of controls was tested using an independent set of samples. Our findings suggest that glycoprotein modifications may be a means to identify novel diagnostic markers for detection of ovarian cancer.

KEYWORDS: biomarkers, ovarian cancer, glycoproteins, lectin arrays, LC–MS/MS

INTRODUCTION

Ovarian cancer is the fifth leading cause of cancer-related death among women in the United States.1 Nearly 22,000 women were diagnosed in 2011 and nearly 16,000 died of their diseases. The poor prognosis in ovarian cancer relates primarily to the fact that ∼70% of ovarian cancer patients present with late stage (stage III/IV) disease. While cure rates for ovarian cancer patients approach >90% when the disease is restricted to the ovaries, long-term survival for patients with late stage disease is less than 30%.2 Thus, diagnostic biomarkers for early stage ovarian cancer are desperately needed.

Currently, serum CA125 (cancer antigen 125) is the most widely used serum marker for diagnosis or prognosis of ovarian cancer. While CA125 is highly effective as a surveillance tool, it is not an effective screening tool. CA125 levels are elevated in only 50 to 60% of women who have early stage ovarian cancer.3 In addition, a number of benign conditions can cause elevation of CA125 levels.4 Thus, CA125 lacks both the sensitivity and specificity to serve as an effective screening tool. However, CA125 is still used for clinical purposes. Numerous efforts have been made to identify novel or complementary biomarkers with improved sensitivity and specificity for detection of ovarian cancer.5 Several promising serum biomarkers have been identified in previous studies, which include human epididymis protein 4 (HE4),6 apolipoprotein A1 (Apo A1),7 B7–H4,8 and transferrin.9 Unfortunately, none of these biomarkers have been effectively used to detect ovarian cancer with sufficient sensitivity and specificity.10 Recently, multimarker panels were developed to discriminate ovarian cancer from controls, but no biomarker combination was found to outperform CA125 alone.5

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Published: July 24, 2012
While a significant effort has been made to identify proteins such as CA125, which are specifically expressed in ovarian cancer cells, recent studies suggest that tumor associated proteins may have unique post-translational modifications and that novel biomarkers may be detectable based upon differences in the protein modifications rather than absolute differences in total protein concentration. Glycosylation is one of the most important post-translational modifications of proteins and plays an important role in regulating protein–protein interactions, cellular recognition, and, in particular, cancer progression. Changes in serum protein glycosylation have been reported to contribute to disease pathogenesis in various cancers such as ovarian cancer, breast cancer, pancreatic cancer, and hepatocellular cancer. At present, a number of serum glycoproteins have been used as therapeutic targets and biomarkers for diagnosis or monitoring the progress of cancers. Some widely used serum glycoprotein biomarkers for diagnosis of cancers include HER2/NEU in breast cancer, α-fetoprotein (AFP) in hepatocellular carcinoma, prostate-specific antigen (PSA) in prostate cancer, and CA125 in ovarian cancer.

Herein, we have developed a lectin-based glycoproteomics strategy to identify serum glycoprotein biomarkers for the detection of ovarian cancer (Figure 1). A lectin array strategy was first applied to detect overall lectin-specific glycosylation changes in serum proteins from patients with ovarian cancer and those with benign conditions. The lectins that showed significantly different responses were used to extract glycoproteins that had been labeled using isobaric chemical tags. The glycoproteins were then identified and quantified by LC–MS/MS and five differentially expressed glycoproteins were identified. The differentially expressed glycoproteins were further verified by lectin-ELISA and ELISA assay. Finally, corticosteroid-binding globulin (CBG), serum amyloid p component (SAP), complement factor B (CFAB) and histidine-rich glycoprotein (HRG) were identified as the most promising markers for differentiating ovarian cancer from benign diseases or healthy controls.

MATERIALS AND METHODS

Clinical Specimens

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. Three different serum banks were analyzed in this study. The first group of samples (discovery) comprised 12 subjects with benign tumors and 22 subjects with stage III ovarian cancers. Samples were collected preoperatively at the University of Michigan as part of an IRB approved tumor banking protocol. Five milliliters of blood were collected by venipuncture directly into serum separator tubes, centrifuged for 12 min, and then aliquoted into polypropylene vials. Frozen aliquots were stored at −70 °C until use.

These samples were used in lectin array analysis and quantitative LC–MS/MS analysis for biomarker discovery. A second serum bank used for initial confirmation consisted of 15 healthy controls, 19 benign ovarian diseases, 21 stage I/II ovarian cancers, and 30 stage III ovarian cancers. All of the healthy controls were provided by the Early Detection Research Network and the Great Lakes-New England CVC (EDRN-GLNE). In the second serum bank, 11 benign and 18 stage III ovarian cancer samples were from the first serum bank; the other 8 benign, 21 stage I/II, and 12 stage III ovarian cancer...
samples were provided by ProteoGenex (Manhattan Beach, CA, USA). The second serum bank contained some of the samples from the discovery set, and thus is not an independent test set. A third serum bank used as an independent confirmation was comprised of 15 healthy controls, 15 benign, 15 stage I/II, and 15 stage III cancer. The 15 healthy controls were from EDRN-GLNE. The 15 benign, 15 stage I/II, and 15 stage III cancer samples were from a sample set provided by Gynecologic Oncology Group (GOG). Benign diseases refer to tumors that do not grow in an aggressive manner and invade surrounding tissues. Stage I/II cancers refer to early stage ovarian cancers where tumors are confined to the ovaries or have spread to pelvic organs. Stage III cancers are late stage ovarian cancers where tumors have spread to abdominal organs or the lymphatic system. Because cancer development and inflammation are inextricably linked, patients with benign tumors and malignant tumors may both have inflammation. Also, patients with benign diseases and patients with ovarian cancers both underwent surgery before serum sample collections, while healthy participants did not suffer from surgery. Thus, in this study, we introduced both benign tumors and normal healthy subjects as controls. A summary of clinical data is given in Table 1.

### Sample Processing

Twelve high abundance serum proteins were removed by a human IgY-12 LC-10 column kit (Beckman Coulter, Brea, CA). The depletion was performed using 250 μL of each serum sample according to the manufacturer’s procedures. The collected flow-through underflow buffer exchange using a YM-3 centrifugal device (Millipore Corp., Bradford, MA), and the sample volume was reduced from 10 mL to 500 μL. The final protein concentration of each sample was measured by the Bradford protein assay kit (Bio-Rad, Hercules, CA).

### Lectin Array

Sixteen lectins with different specificities (Supplemental Table S1) were printed on nitrocellulose coated glass slides (Avid, Grace Bio-Laboratories) using a piezoelectric noncontact printer (Nano plotter, GeSim). The concentration of each lectin was 1 mg/mL in 10% PBS, and each lectin was printed in triplicate. After printing, the slides were blocked with 1% BSA in PBS and washed three times with PBST (0.1% Tween 20 in PBS). Ten micrograms of protein from each depleted serum sample were reduced by 5 mM TCEP for 30 min. The reduced proteins were labeled by EZ-link iodoacetyl-LC-biotin (Pierce) for 1.5 h. The labeled serum samples were hybridized with the slides for 1 h followed by incubation with streptavidin conjugated fluorescent dye (Alexa555, Invitrogen Biotechnology) for 1 h. The signal intensity was detected by a microarray fluorescent scanner (Genepix 4000A, Axon).

### Exactag Isobaric Labeling and Lectin Extraction of Glycoproteins

One hundred micrograms of protein from each depleted serum sample from 12 benign and 22 ovarian stage III cancers were labeled with the Exactag isobaric labeling reagents (PerkinElmer) as previously described. After mixing together labeled samples, the buffer was exchanged to lectin binding buffer (1× PBS, 1 mM MgCl₂, and 1 mM MnCl₂) using Ultrasil YM-3. Agarose-bound LCA and UEAI, which were selected based on the lectin array experiment, were individually used to extract glycoproteins. Columns packed with 1 mL of LCA or UEAI were washed and equilibrated with 3 mL of binding buffer. Two hundred micrograms of Exactag labeled proteins in 1 mL of binding buffer were loaded onto the column and incubated for 15 min. The column was washed with 5 volumes of binding buffer and then the captured glycoproteins were eluted with 4 volumes of elution buffer (0.1 M α-methyl mannoside in PBS for LCA and 100 mM t-fucose in PBS for UEAI). Each sample was concentrated using Microcon YM-3 to 200 μL in 25 mM NH₄HCO₃.

### Mass Spectrometry

The labeled and extracted glycoproteins were digested with trypsin at 37 °C overnight. N-Glycans were released from asparagine (Asn) residues using PNGase F (New England Biolabs, Ipswich, MA). The resulting peptides were analyzed by LC–MS/MS in an LTQ mass spectrometer according to a previously described method. Briefly, the nano-RPLC column (Nano Trap column, 5 μm 200 Å Magic C18AQ 100 μm × 150 mm, Michrom Bioresources, Auburn, CA) was directly coupled to an LTQ linear IT MS from Thermo Scientific (Waltham, MA) with a nanospray source. The LTQ instrument was operated in positive ion mode. The spray voltage was set at 2.5 kV and the capillary voltage at 30 V. The ion activation was achieved by utilizing helium at a normalized collision energy of 35%. The scan range of each full MS scan was m/z 400–2000. Acetonitrile gradients of 5–35% for 60 min at a flow rate of 300 nL/min were applied for the separation of peptides as previously described. For detection, the MS was set as a full scan followed by three data-dependent MS2 events. A 1 min dynamic exclusion window was applied. All MS/MS spectra were searched against the IPI database (IPI hu-
The search was performed using SEQUEST (version 27) incorporated in Proteome Discover software version 1.1 (Thermo Scientific). The search parameters were as follows: (1) fixed modification, carbamidomethylation of C; (2) variable modifications, oxidation of M; (3) variable modifications, asparagine (Asn) to aspartate (Asp) conversion after PNGase F treatment (+0.984 Da); (4) allowing two missed cleavages; (5) precursor ion mass tolerance, 1.4 Da; (6) fragment ion mass tolerance, 1.5 Da. The protein identification result was filtered using a 1% false discovery rate cutoff. The Exactag analysis software 3.0 (PerkinElmer) was applied to quantitatively analyze the protein abundance.

In-Plate Lectin-ELISA Assay

In-plate lectin-ELISA assay was performed as described previously with some modifications. Briefly, 100 μL of monoclonal antibodies were added to each well of a 96-well ELISA plate and incubated at 37 °C for 1 h. The antibodies coated on the plates were oxidized by 200 mM NaIO₄ at 4 °C for 5 h and derivatized with 1 mM MPBH and 1 mM Cys-Gly overnight. The plate was then blocked with 3% BSA in PBST (0.1% Tween-20 in PBS) for 1 h. Serum samples were diluted 50-fold with 0.1% Brij in PBST. One hundred microliters of each diluted serum sample was applied to each well of a 96-well ELISA plate. After 1 h incubation, the plate was rinsed with PBST five times to remove unbound proteins. One hundred microliters of biotinylated AAL (1 μg/mL) was added to bind with fucosylated antigens. HRP-conjugated streptavidin was then applied to each well followed by TMB working solution and stop solution. To determine the concentration of the fucosylated CBG, SAP, CFAB, and HRG, the absorbance at 450 nm of the plate was measured.

Monoclonal antibodies used for lectin-ELISA assay include mouse anti-corticosteroid-binding globulin, mouse antiserum amyloid P-component, mouse anti-histidine-rich glycoprotein, and mouse anticomplement factor B. All the antibodies were purchased from Abcam (Cambridge, MA, USA).

ELISA Assay

The underlying protein abundances of CBG, SAP, CFAB, and HRG were measured by ELISA assay. The CA125 level was also measured in this study. ELISA kit for CBG was purchased

Figure 2. Overall glycosylation levels detected by lectin array. (a) Response of 16 different lectins to glycoproteins in depleted serum from ovarian cancer stage III and benign patients. (b) Three lectins with fucosylation-binding specificity (LCA, UEAI, and AAL) can differentiate ovarian cancer stage III samples from benign samples (*, p < 0.05). (c) SNA with NeuSAcr2-6Gal (NAc)-R binding specificity can differentiate ovarian cancer stage III samples from benign samples (**, p < 0.01).
Table 2. Significantly Changed Proteins between Stage III Ovarian Cancer and Benign Diseases Detected by Exact Tag Labeling Based Quantitative LC–MS/MS Analysis

<table>
<thead>
<tr>
<th>protein ID</th>
<th>protein name</th>
<th>p value*</th>
<th>lectin affinity</th>
<th>function</th>
</tr>
</thead>
<tbody>
<tr>
<td>P08185</td>
<td>corticosteroid-binding globulin</td>
<td>0.0027</td>
<td>LCA</td>
<td>major transport protein for glucocorticoids and progestins in the blood</td>
</tr>
<tr>
<td>P04196</td>
<td>histidine-rich glycoprotein</td>
<td>0.05</td>
<td>LCA</td>
<td>cell surface binding</td>
</tr>
<tr>
<td>P00751</td>
<td>complement factor B</td>
<td>0.05</td>
<td>LCA</td>
<td>part of complemen system</td>
</tr>
<tr>
<td>Q66FD5</td>
<td>N-acetylmuramoyl-t-dalanine amidase</td>
<td>0.018</td>
<td>UEAI</td>
<td>may play a scavenger role by digesting biologically active peptidoglycan into biologically inactive fragments</td>
</tr>
<tr>
<td>P05543</td>
<td>thyroxine-binding globulin</td>
<td>0.007</td>
<td>UEAI</td>
<td>major thyroid hormone transport protein in serum</td>
</tr>
</tbody>
</table>

*p value: statistical significance of changed proteins between ovarian cancer and benign diseases after Student's t-test.

Table 3. Summary of Protein Fucosylation and Their Underlying Protein Concentrations Showing Potentially Significant Differences in Ovarian Cancer Serum Samples by AAL-ELISA or ELISA Assay Using Confirmatory Sample Set I

<table>
<thead>
<tr>
<th>protein</th>
<th>fucosylated protein levels (μg/mL)</th>
<th>underlying protein concentrations (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean (range)</td>
<td>mean (range)</td>
</tr>
<tr>
<td>CBG</td>
<td>stage III 1.80 (0.78–2.94)</td>
<td>stage III 17.1 (11.2–24.7)</td>
</tr>
<tr>
<td></td>
<td>stage I/II 1.51 (0.64–3.89)</td>
<td>stage I/II 19.0 (12.3–25.3)</td>
</tr>
<tr>
<td></td>
<td>benign 1.65 (0.81–3.44)</td>
<td>benign 19.3 (12.6–28.8)</td>
</tr>
<tr>
<td></td>
<td>normal 1.65 (0.81–3.44)</td>
<td>normal 22.6 (17.8–36.8)</td>
</tr>
<tr>
<td>SAP</td>
<td>2.44 (1.12–3.81)</td>
<td>259.2 (67.9–400.0)</td>
</tr>
<tr>
<td></td>
<td>2.1 (0.75–4.0)</td>
<td>204.4 (7.6–358.7)</td>
</tr>
<tr>
<td></td>
<td>1.9 (0.96–3.85)</td>
<td>199.2 (36.0–368.0)</td>
</tr>
<tr>
<td>HRG</td>
<td>2.31 (1.11–3.64)</td>
<td>15.8 (6.9–30.9)</td>
</tr>
<tr>
<td></td>
<td>1.98 (0.72–4.0)</td>
<td>17.4 (7.8–37.6)</td>
</tr>
<tr>
<td></td>
<td>1.63 (0.87–3.67)</td>
<td>13.5 (7.8–41.8)</td>
</tr>
<tr>
<td>CFAB</td>
<td>3.0 (1.61–4.0)</td>
<td>29.4 (3.3–106.8)</td>
</tr>
<tr>
<td></td>
<td>2.79 (0.92–4.0)</td>
<td>39.4 (6.9–125.8)</td>
</tr>
<tr>
<td></td>
<td>2.17 (1.02–4.0)</td>
<td>45.6 (5.9–144.8)</td>
</tr>
<tr>
<td></td>
<td>2.35 (1.02–4.0)</td>
<td>37.5 (16.0–60.3)</td>
</tr>
</tbody>
</table>

*p value: statistical significance of changed proteins between ovarian cancer and benign diseases after Student's t-test.

Statistical Analysis

All statistical analyses were performed using SPSS 11.5. Statistical differences were determined using the Student's t-test that assumes Gaussian distribution. The Wilcoxon rank-sum test was used for pairwise comparison of markers between various disease groups when the population data does not follow the Gaussian distribution. For all statistical comparisons, p < 0.05 was taken as statistically significant. Receiver operating characteristic (ROC) curves were produced in terms of the sensitivity and specificity of markers at their specific cutoff values. Multivariate analysis was also done by logistic regression to find the best-fitting multivariate model for each comparison group.

Results

Overall Glycosylation Changes Detected by Lectin Array

We used a lectin array to identify differential glycoprotein binding to a panel of lectins. We screened 16 lectins for differential binding to sera samples from patients with benign diseases and patients with stage III ovarian cancer (Figure 2a). After Student’s t-test analysis, LCA, UEAI, and SNA (p < 0.05) lectins showed a significant differential response between cancer and benign. The lectin AAL also showed a difference in binding to sera samples from patients with benign cancer and benign benign. The lectin AAL also showed a differential response between stage III and benign/normal (fucosylation); for differential response between stage III/stage I/II and normal, and stage III/stage I/II and benign (underlying protein).

Figure 3. Fucosylated protein alterations confirmed by AAL-ELISA. Comparison of response intensity of CBG, SAP, HRG, and CFAB to AAL in normal healthy controls, benign diseases, stage I/II ovarian cancer, and stage III ovarian cancer. The fucosylated protein levels with significant changes between pairwise comparisons are indicated (p < 0.05).
specificity to fucose, while SNA prefers to capture the NeuSAct2-6Gal (NAc)-R structure (Figure 2c).

**Discovery of Novel Glycoprotein Biomarkers by Quantitative LC–MS/MS**

To identify glycosylation changes in the serum of ovarian cancer patients, we labeled proteins using Exactag isobaric tags and extracted the labeled glycoproteins using a lectin column. The proteins were then digested and analyzed by LC–MS/MS. This method allowed us to quantify the expression of glycoproteins between the cancer and benign samples. On the basis of the lectin-array results, we used LCA and UEAI to separately extract the fucosylated proteins and performed a quantitative proteomics analysis. As a result, five proteins were identified to display abnormal expression levels in ovarian cancer. Among them, CBG, HRG, and CFAB were extracted from LCA, and PGRP2 and THBG were from UEA-I as shown in Table 2. Among the five significantly changed glycoproteins (p < 0.05 after Student’s t-test), HRG and CFAB have higher intensity in cancer samples than benign, while CBG, PGRP2, and THBG are detected with higher levels in benign samples. An MS/MS spectrum of a peptide from corticosteroid-binding globulin is shown in Supplemental Figure S1.

![Figure 4 ROC curve for significantly changed fucosylated CBG, SAP, HRG, and CFAB detected by AAL-ELISA assay.](image)

(a) ROC curve for differentiating stage III ovarian cancer from benign diseases; (b) ROC curve for fucosylated CFAB to distinguish early stage cancer from benign diseases or healthy controls.

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*Journal of Proteome Research*

dx.doi.org/10.1021/pr3003302/J. Proteome Res. 2012, 11, 4541–4552
Confirmation of Fucosylation Change of Protein by Lectin-ELISA Assay

Lectin-ELISA assay allows differentiation based upon changes in the glycosylation profile of a specific protein, rather than the total protein abundance. From the quantitative results of mass spec analysis, we identified three novel candidate biomarkers, CBG, HRG, and CFAB, for detection of ovarian cancer and found that their fucosylation levels were significantly changed in ovarian cancer. An AAL-ELISA assay was thus employed to confirm the fucosylation changes of these candidates from the crude serum samples without depletion of high abundance proteins (Table 3). AAL was used instead of LCA or UEAI for the lectin-ELISA assay since it had a superior performance with the lectin-ELISA assay compared to the other lectins.

The Student’s t-test was applied to analyze the variance of protein response to AAL in benign and stage III cancer serum samples. CFAB and HRG showed significantly higher response in stage III ovarian cancer sera than in the benign sera ($p < 0.05$, Figure 3), which is consistent with LC−MS/MS quantification. CBG, which was found to be decreased in the stage III cancer sera by Exactag labeling based quantitative LC−MS/MS analysis, showed an increase in cancer sera in the AAL-ELISA assay. An ROC curve was constructed for each of the four changed fucosylated proteins to distinguish stage III cancer from benign diseases as shown in Figure 4a. The AUC for CBG, SAP, CFAB, and HRG was 0.733, 0.750, 0.740, and 0.733, respectively.

For the AAL-ELISA assay, we also measured the abundance of the fucosylated CBG, SAP, CFAB, and HRG in healthy controls and patients with early stage (stage I/II) ovarian cancer. Compared to healthy controls, the fucosylation of the above four proteins were all significantly increased in stage III cancer samples ($p < 0.05$, Figure 3). We further found that fucosylated CFAB was increased in early stage ovarian cancer samples.
compared to benign diseases or healthy controls ($p < 0.05$, Figure 3). The AUC for fucosylated CFAB to distinguish early stage ovarian cancer from benign diseases or healthy controls was 0.679 and 0.651, respectively, as shown in Figure 4b.

We evaluated an independent set of samples from GOG to verify the fucosylation changes we observed. We confirmed the increased fucosylation of CBG, SAP, HRG, and CFAB in stage III ovarian cancer compared to benign diseases or healthy controls (Figure 5a). Importantly, we verified the increased fucosylation of CFAB in early stage cancer. The AUC for fucosylated CFAB to distinguish early stage ovarian cancer from benign diseases or healthy controls was 0.773 and 0.791 respectively, as shown in Figure 5b. Furthermore, by using this sample set, we also found that fucosylated CBG, SAP, and HRG were increased in early stage cancer compared to benign diseases or healthy controls (Figure 5a). The ROC curves for fucosylated CBG, SAP, and HRG to distinguish early stage cancer from benign diseases or healthy controls are shown in Supplemental Figure S2.

**Underlying Protein Level Changes Detected by ELISA Assays**

Since alterations in the expression of the underlying protein could account for the detected glycosylation changes, we used ELISA assays to measure the underlying protein concentrations of CBG, SAP, CFAB, and HRG in the serum samples from the initial confirmation sample set. Among the four proteins measured by ELISA, CFAB had no statistically significant changes between ovarian cancer and healthy controls or benign diseases ($p > 0.05$ for all pairwise comparisons), whereas total abundance of CBG and HRG consistently decreased from healthy controls to stage III cancer, and protein levels of SAP were increased in cancer (Figure 6). CA125 level changes are also shown (Supplemental Figure S3).

ROC curves for CBG, SAP, and HRG were constructed to differentiate stage III ovarian cancer from healthy controls and also found that SAP was significantly decreased in ovarian cancer compared to benign diseases or healthy controls, as shown in Figure 8b. Importantly, we used an independent serum bank from the GOG to verify the protein level changes of CBG and SAP in ovarian cancer. We confirmed that the protein level of CBG was significantly decreased in ovarian cancer compared to benign diseases or healthy controls, while SAP was significantly increased (Figure 8a). We also confirmed that the combination of CBG and SAP had an AUC of 0.862, which showed comparable performance to CA125 to differentiate stage III cancer from benign disease (Figure 8b). Importantly, we verified the performance of CBG in differentiating early stage ovarian cancer from healthy controls and also found that SAP could be effectively used to distinguish early stage cancer from benign diseases or healthy controls, as shown in Figure 8b.

**DISCUSSION**

In serum, approximately 50% of proteins are glycosylated where a diversity of glycans and glycoprotein alterations have been reported to be involved in the development and progression of cancers. Thus, alterations in glycoprotein modifications may serve as potential cancer biomarkers. The isotope tag labeling approach provides a method to compare the relative peptide abundances directly and enables accurate quantitative results. The isotope tag reagent Exactag used in our studies is able to label up to 10 samples simultaneously, which makes it advantageous in analyzing a large number of clinical samples. In the present study, we used lectin arrays to detect overall lectin-specific glycosylation changes of depleted serum between
stage III ovarian cancer and benign diseases. We found that the levels of protein fucosylation and sialylation were significantly changed between stage III cancers and benign, while other types of glycosylation showed no significant changes. As fucosylation changes have been reported in a number of cancers and are regarded as promising targets of cancer diagnosis and therapy, we mainly focus on analyzing fucosylation changes in ovarian cancer.

It has been reported that both the expression and activity of fucosyltransferases are increased in ovarian cancers. Several fucosylated proteins have already been found up-regulated in ovarian cancer, which include alpha-1-antitrypsin, haptoglobin, and alpha-1-proteinase inhibitor. In the present work, we have identified the fucosylation changes of CBG, HRG, and CFAB in the serum of ovarian cancer patients. However, we did not identify the clinically used biomarker CA125 by LC–MS/MS. This may be due to the low abundance of CA125 (ng/mL) and the limited sensitivity of mass spectrometric detection. By quantitative LC–MS/MS analysis, we found that fucosylated CBG was decreased in ovarian cancer, while by AAL-ELISA assay, we found its fucosylation levels were increased. This difference may be due to the use of lectin LCA to extract fucosylated glycoproteins before quantification and identification by LC–MS/MS, while the lectin AAL was used for confirmation of the fucosylation changes by lectin-ELISA.

For the lectin-ELISA assay, it was critical to block the binding between lectins and the glycans of the spotted antibodies. We used sodium periodate (NaIO₄) to oxidize the glycans of the antibodies before reaction with lectins. For AAL, the average intensity of the assays incubated with serum was about 20 times stronger than the average intensity of the assays incubated with PBS after oxidation, while for LCA and UEAI, there was less than 2-fold difference (Supplemental Figure S4). As LCA or

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**Figure 7.** ROC curve analyses of significantly changed proteins detected by ELISA. (a) ROC curve for differentiating stage III ovarian cancer from normal healthy controls; (b) ROC curve for differentiating stage III ovarian cancer from benign diseases; (c) ROC curve for distinguishing stage I/II ovarian cancer from normal healthy controls.
UEAI could not be readily used in the lectin-ELISA assay, AAL was used instead. Although lectin AAL and LCA both bind to fucosylated glycoproteins, the glycan structures they recognize are different. AAL recognizes R1-3/R1-4 and R1-6 fucose, while LCA specifically recognizes core fucose of N-glycans. This may account for the different fucosylated CBG levels between quantitative LC~MS/MS analysis and the AAL-ELISA assay.

We also examined the fucosylation changes of serum amyloid P-component by AAL-ELISA and found that fucosylated SAP was increased in ovarian cancer. Serum amyloid P-component is a member of the pentraxins family and has 51% sequence homology with C-reactive protein, which is a classical acute phase response plasma protein. C-reactive protein has been reported to be associated with ovarian cancer. However, the expression of SAP in plasma of ovarian cancer patients has not been studied. Furthermore, in our previous study, glycosylation of SAP was found significantly changed in pancreatic cancer. Thus, SAP was also included in our biomarker testing.

Changes in protein glycosylation may result from alterations in the underlying protein concentration or from actual glycosylation level changes. In order to evaluate this effect, ELISA assays were used to determine the underlying protein concentrations from the original serum samples. Interestingly, we found that protein levels of CBG were decreased in ovarian cancer, while its fucosylation levels were increased. The opposing trend of underlying protein levels and fucosylation levels may indicate a dramatically aberrant fucosylation of CBG in ovarian cancer. This may be biologically relevant as CBG is a plasma glycoprotein that binds steroid hormones such as progesterone and cortisol, which have been implicated in the progression of ovarian cancer. The consistent decrease of the CBG level that occurs from healthy controls to stage III cancer as well as its aberrant fucosylation in cancer could indicate that CBG may be a potential candidate biomarker for detection of ovarian cancer.

CFAB, a component of the alternative pathway of the complement system, circulates in the blood as a single N-glycosylated polypeptide chain. The protein level of CFAB has been reported to be increased in the sera of patients with breast cancer and pancreatic cancer. However, the roles of CFAB in the progress of cancer are not well understood. In the present study, we identified and confirmed that fucosylated
CFAB levels were increased in ovarian cancer, while the total protein levels of CFAB remained the same between controls and ovarian cancer. From the results, we can conclude that fucosylation changes and protein level changes could be complementary to each other and effectively used to detect ovarian cancer.

Because of the limited performance of CA125 in detecting early stage ovarian cancer, it is important to identify candidate biomarkers that could be effectively used to detect early stage ovarian cancers. In addition, since benign diseases may cause elevation of CA125 levels, it is essential to identify complementary candidate biomarkers to CA125 in order to improve its performance in differentiating ovarian cancer from benign diseases. In this study, we found that the protein level changes in CBG and HRG can be used to distinguish early stage ovarian cancer from healthy controls. Furthermore, fucosylated CFAB could be used to differentiate early stage cancer from benign diseases or healthy controls. We found that the combination of CBG and SAP showed comparable performance to CA125 in distinguishing stage III cancer from benign diseases. Moreover, the combination of CBG, SAP, and CA125 improved both the sensitivity and specificity in distinguishing stage III ovarian cancer from benign diseases compared to CA125 alone.

**CONCLUSIONS**

We applied a comprehensive strategy to identify and confirm fucosylated glycoprotein biomarkers for the detection of ovarian cancer. Our results suggest that changes in protein levels or alterations of fucosylation levels could both be effectively used for detecting ovarian cancer. Finally, SAP, CBG, HRG, and CFAB were identified as the most promising candidate markers to supplement CA125 for detecting ovarian cancer. These studies suggest a novel means to identify diagnostic biomarkers for ovarian cancer.

**ASSOCIATED CONTENT**

**Supporting Information**

Sixteen lectins used for lectin array and their specificities; Exactag labeled samples; total proteins identified from the LCA and UEAI fractions; MS/MS sequencing data of a glycopeptide from corticosteroid-binding globulin identified in the LCA extracted fractions; ROC curves for fucosylated CBG, HRG, and SAP to distinguish early stage ovarian cancer from benign diseases or healthy controls by using an independent confirmation sample set from GOG; CA125 levels measured from confirmation sample set I and confirmation sample set II; curves for antibody SAP response to different dilutions of serum with AAL, LCA, and UEAI lectin detection. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS USED**

CA125, cancer antigen 125; CBG, corticosteroid-binding globulin; SAP, serum amyloid P-component; HRG, histidine-rich glycoprotein; CA125, complement factor B; PGRP2, N-acetylmuramoyl-L-alanine amidase; THBG, thyroxine-binding globulin; LCA, *Lens culinaris* agglutinin; UEAI, ulex europaeus agglutinin I; SNA, *Sambucus nigra* lectin; LTQ MS/MS, linear ion trap tandem mass spectrometry

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