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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

15 APR 2010 - 14 APR 2014

Abstract
Profiling of the glycan structures that differ between the serum of serous ovarian cancer and benign conditions have been performed using lectin array 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 have evaluated 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, normals, early stage ovarian cancer and stage 3c serous ovarian cancer. These glycoproteins have been used for further blinded validations using a limited set.

Subject Terms
Glycoproteins, Biomarkers, lectins, microarrays
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Introduction: In our previous work(1-3), using an integrated platform containing mass spectrometry and lectin microarray, a panel of glycoprotein biomarkers has been identified for detection of ovarian cancer, where some of the candidate markers were found to show the potential to detect early stage ovarian cancer. As a follow up in the current year we sought to evaluate the performance of discovered biomarker candidates LRG1, HRG, CLUS, CBG, and SAP in differentiating ovarian cancer from healthy controls or non-cases, and to test if these markers can be complementary with CA125 to improve ovarian cancer detection with two independent blinded sample sets, which include healthy controls, surgical controls, benign controls, early stage ovarian cancer and late stage ovarian cancer provided by the POCRC.

Description of Progress: We have been working according to the SOW and have accomplished most of the work in the original proposal. In the current year we have been performing blinded validations on our markers using ELISA assays mainly on the protein level.

Patient Population and Study Design: Two serum banks provided by the Pacific Ovarian Cancer Research Consortium(POCRC) were analyzed in this study. The first sample set for the preliminary verification is composed of 156 serum samples including 36 healthy controls, 16 surgical normal, 43 benign diseases, 19 stage I/II cancer, and 42 stage III/IV cancer. The second sample set, a larger blinded confirmation set, is composed of 233 patients including 77 healthy controls, 32 surgical normal, 53 benign diseases, 27 stage I/II cancer, and 44 stage III/IV cancer. The first set was performed blinded and then unblinded for us, whereas sample set 2 was never unblinded. The statistical analysis for the sample set 2 was done by a statistician from the POCRC.

Results: In previous work, we found the abnormal expression of HRG, CLUS, LRG1, CBG, and SAP in ovarian cancer(1-3). Herein, we further verified our findings using two independent blinded sample sets to explore the most promising candidate markers or marker panels to detect ovarian cancer with improved sensitivity and specificity. All the assays were performed blinded to clinical data.

Figure 1. ROC analyses for CA125, LRG1, HRG, CLUS, and CBG to differentiate ovarian cancer from non-cases (a) and early stage cancer from non-cases (b).

We studied the distribution of marker values by ovarian cancers and non-cases in 156 serum samples from the preliminary confirmation set 1. HRG, CLUS, and CBG significantly decreased in ovarian cancer patients compared with non-cases, while LRG1 and SAP showed increased expression in cancers. HRG decreased from a median of 186.3 μg/mL (range, 39.95–384.3 μg/mL) in non-cases to 140.4 μg/mL (range, 31.3–73.8 μg/mL) in ovarian cancer patients, while LRG1 increased from a median of 15.51 μg/mL (range, 3.06–93.32 μg/mL) in non-cases to 24.92 μg/mL (range, 5.66–93.32 μg/mL) in ovarian cancer patients. Consistent with established results, the clinically used marker of CA125 increased from healthy controls to benign disease to ovarian cancer (p<0.01 for all pairwise comparisons). Altered expression of
HRG, LRG1, CLUS, CBG, and SAP was further validated using an independent larger blinded sample set 2. Consistent with the results using sample set 1, HRG, CLUS, and CBG significantly decreased in ovarian cancer patients, while LRG1 and SAP showed increased expression in cancers compared with non-cases.

ROC curves for the six markers for distinguishing ovarian cancer (early stage and late stage cancer) from non-cases (healthy, surgical, and benign controls) are shown in Figure 1a. CA125 obtained the highest AUC (0.908), followed by LRG1 (AUC=0.787) for differentiating ovarian cancer from non-cases. The AUC values for HRG, CLUS, SAP, and CBG were 0.713, 0.716, 0.645, and 0.621, respectively. By the optimal cutoff that maximizes the sensitivity+specificity, CA125 provided a sensitivity of 80% and specificity of 98% at a cut-off of 81 U/mL, while LRG1 showed a sensitivity of 70% and specificity of 76% at a cut-off of 18.75 μg/mL to differentiate ovarian cancer from non-cases. To distinguish early stage cancer from non-cases, CA125 achieved the highest AUC of 0.812. CBG (AUC=0.764) and HRG (AUC=0.746) showed comparable ability to CA125 in differentiating early stage cancer from non-cases. The AUC value for LRG1 is 0.707 (Figure 1b). The performance of candidate markers in differentiating ovarian cancer from non-cases was further confirmed using the independent blinded sample set 2. Consistent with the results from sample set 1, CA125 obtained the highest AUC of 0.915, followed by LRG1 (AUC=0.786) for differentiating ovarian cancer from non-cases. The AUC for HRG, CLUS, and CBG was 0.768, 0.748, and 0.694, respectively.

The performance of candidate markers in differentiating ovarian cancer from non-cases was further confirmed using the independent blinded sample set 2. The AUC values for CA125, LRG1, HRG, CLUS, and CBG to distinguish ovarian cancer from healthy controls were 0.897, 0.772, 0.740, 0.807, and 0.693, respectively (Figure 2a). By the optimal cutoff that maximizes the sensitivity+specificity, CA125 provided a sensitivity of 86% and specificity of 90% at a cut-off of 30.9 U/mL, while LRG1 showed a sensitivity of 57% and specificity of 90% at a cut-off of 23.64 μg/mL to differentiate ovarian cancer from healthy controls. HRG, CLUS, and CBG also showed good performance to differentiate early stage ovarian cancer from healthy controls as shown in Figure 2b. The AUC values for CA125, HRG1, CLUS, CBG, and LRG1 were 0.802, 0.781, 0.759, 0.843, and 0.685, respectively. The performance of candidate markers of LRG1, HRG, CLUS, and CLUS for discriminating ovarian cancer from healthy controls was also confirmed by the larger blinded sample set 2. HRG (AUC=0.885) and CLUS (AUC=0.870) showed comparable performance to CA125 (AUC=0.938) for distinguishing cancer from healthy controls. At a 10% false-positive rate, CA125 detects 88.7% cancers, 74.6% and 66.2% for CLUS and HRG1. The AUC values for CBG and LRG1 were 0.807 and 0.794, respectively.

The AUC values for CA125, LRG1, HRG, CLUS, and CBG to distinguish ovarian cancer from early stage cancer and healthy controls were 0.897, 0.772, 0.740, 0.807, and 0.693, respectively (Figure 2a). By the optimal cutoff that maximizes the sensitivity+specificity, CA125 provided a sensitivity of 86% and specificity of 90% at a cut-off of 30.9 U/mL, while LRG1 showed a sensitivity of 57% and specificity of 90% at a cut-off of 23.64 μg/mL to differentiate ovarian cancer from healthy controls. HRG, CLUS, and CBG also showed good performance to differentiate early stage ovarian cancer from healthy controls as shown in Figure 2b. The AUC values for CA125, HRG1, CLUS, CBG, and LRG1 were 0.802, 0.781, 0.759, 0.843, and 0.685, respectively. The performance of candidate markers of LRG1, HRG, CLUS, and CLUS for discriminating ovarian cancer from healthy controls was also confirmed by the larger blinded sample set 2. HRG (AUC=0.885) and CLUS (AUC=0.870) showed comparable performance to CA125 (AUC=0.938) for distinguishing cancer from healthy controls. At a 10% false-positive rate, CA125 detects 88.7% cancers, 74.6% and 66.2% for CLUS and HRG1. The AUC values for CBG and LRG1 were 0.807 and 0.794, respectively.
Multivariate analysis was done by logistic regression to find the best-fitting multivariate model for each comparison group. Using sample set 1, we found the combination of LRG1 and CA125 resulted in an AUC of 0.916 (OR LRG1=1.048, p=0.012; OR CA125=1.006, p=0.001), which outperforms the performance of CA125 (AUC=0.908) (LR test p=0.02) in distinguishing ovarian cancer from non-cases, indicating that LRG1 may provide independent diagnostic value in addition to CA125. The combination of CA125, LRG1, HRG, CLUS, and CBG resulted in an AUC of 0.915, as shown in Figure 3a. To distinguish ovarian cancer from healthy controls, the combination of LRG1, CLUS, HRG, and CBG resulted in an AUC of 0.926, which outperforms CA125 (AUC=0.897). Furthermore, the combination of CA125, LRG1, CLUS, HRG, and CBG resulted in an AUC of 0.949 with a specificity of 97% at a sensitivity of 75%, which showed improved performance in distinguishing ovarian cancer from healthy controls compared to CA125 alone (Figure 3b).

![Figure 3. The performance of multimarker panels in distinguishing ovarian cancer from non-cases or healthy controls using sample set 1.](image)

In summary, using two blinded independent sample sets, LRG1, HRG, CLUS, and CBG were found to be promising candidate markers for detection of ovarian cancer. These markers also show the potential to become useful biomarkers for the diagnosis of early ovarian cancer. The combination of LRG1, HRG, CLUS, and CBG showed improved performance to distinguish ovarian cancer from healthy controls compared to CA125. The performance of these candidate markers for detection of ovarian cancer needs to be further validated using a larger sample set before clinical use.

Key Research Accomplishments:
- A potential panel of markers for distinguishing stage III serous cancer in serum from normal controls or benign disease
- A potential panel of several markers for distinguishing stage I/II serous cancer in serum from normal or benign samples

Reportable Outcomes: Postdoctorals Xiaolei Xie and Jing Wu have been trained in marker studies, ELISAs, lectin-ELISAs and mass spectrometry. Their work has identified a potential panel of markers of ovarian cancer for further confirmation and validation.


Publications:

Meeting Abstracts and Presentations:
Identification and Confirmation of Serum Glycoprotein Biomarkers for Detection of Ovarian Cancer, Jing Wu, Xiaolei Xie, Yashu Liu, Jintang He, Ricardo Benitez, and David M. Lubman, Presented at the HPLC Meeting 2012, Anaheim CA.
Identification of ovarian cancer biomarkers using lectin microarray and Glycoproteomics, Xiaolei Xie, Jing Wu, Yashu Liu, Jintang He, David M. Lubman, Presented at the 2011 ASMS Conference on Mass Spectrometry and Allied Topics.
Identification and Confirmation of Serum Glycoprotein Biomarkers for Detection of Ovarian Cancer, Jing Wu, Xiaolei Xie, Yashu Liu, Jintang He, Ricardo Benitez, Ronald J. Buckanovich and David M. Lubman, Presented at the 2012 ASMS Conference on Mass Spectrometry and Allied Topics. Altered Expression of Sialylated Glycoproteins in Ovarian Cancer Using a Lectin Array and LC-MS/MS, Jing Wu, Xiaolei Xie, Song Nie, Ronald J. Buckanovich and David M. Lubman, Presented at the 2013 ASMS Conference on Mass Spectrometry and Allied Topics.

Conclusion: There are significant changes in fucosylation and sialylation levels between ovarian cancer samples of early and late stage and benign conditions which can be used with lectin columns to extract the proteins responsible for these changes. These proteins can then be further profiled as potential markers for ovarian cancer. We have identified 5-6 potential markers of ovarian cancer that are candidates for further validation that can discriminate early stage ovarian cancer from benign or normal controls. Some of these proteins are known to be related to processes that occur in the progression of cancer. We have performed two sets of blinded validations on these markers and found that no one marker could perform as well as CA125 to discriminate ovarian cancer from benign or normal controls. However, combinations of some of these markers could perform comparable to or show improved performance relative to CA125 or could combine with CA125 to improve its performance for detection of early stage ovarian cancer versus benign or normal controls. This initial blinded validation was with relatively small sets and much larger validation sets will be needed to confirm these results.

References:
Altered Expression of Sialylated Glycoproteins in Ovarian Cancer Sera Using Lectin-based ELISA Assay and Quantitative Glycoproteomics Analysis

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†Department of Surgery, University of Michigan, Ann Arbor, Michigan 48109, United States
‡Caprion Proteomics US LLC, Menlo Park, California 94025, United States
§Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan 48109, United States

ABSTRACT: Herein, we identify and confirm differentially expressed sialoglycoproteins in the serum of patients with ovarian cancer. On the basis of Sambucus nigra (SNA) lectin enrichment and on an isobaric chemical labeling quantitative strategy, clusterin (CLUS), leucine-rich alpha-2-glycoprotein (LRG1), hemopexin (HEMO), vitamin D-binding protein (VDB), and complement factor H (CFH) were found to be differentially expressed in the serum of patients with ovarian cancer compared to benign diseases. The abnormal sialylation levels of CLUS, CFH, and HEMO in serum of ovarian cancer patients were verified by a lectin-based ELISA assay. ELISA assays were further applied to measure total protein level changes of these glycoproteins. Protein levels of CLUS were found to be down-regulated in the serum of ovarian cancer patients, while protein levels of LRG1 were increased. 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. An independent sample set was further used to confirm the ability of these candidate markers to detect patients with ovarian cancer. Our study provides a comprehensive strategy for the identification of candidate biomarkers that show the potential for diagnosis of ovarian cancer. Further studies using a large number of samples are necessary to validate the utility of this panel of proteins.

KEYWORDS: ovarian cancer, biomarkers, glycoprotein, lectin-based ELISA, mass spectrometry

INTRODUCTION

Despite progress in cancer therapy and increasing knowledge of the molecular mechanisms of cancer metastasis, overall survival rates for patients with ovarian cancer have remained virtually unchanged over the past few decades.1 One major reason is the lack of an early detection test for ovarian cancer. Serum CA125 is currently the best marker for detection of ovarian cancer but is not a reliable marker for diagnosing early stage cancers. Thus, diagnostic biomarkers for early stage ovarian cancer are desperately needed. In addition, a number of benign conditions, including pregnancy, endometriosis, normal menstruation, and pelvic inflammatory disease, can cause elevation of CA125 levels.2 Therefore, it is essential to identify complementary candidate biomarkers to CA125 in order to improve its performance in differentiating ovarian cancer from benign diseases.

In recent years, dozens of potential serum biomarkers for detection of ovarian cancer have been identified and been evaluated, including Apo A1,3 HE4,4 epidermal growth factor receptor,5 leptin,6 afamin,7 and C-reactive protein.8 However, none of these candidate biomarkers have been used to detect ovarian cancer with sufficient sensitivity and specificity. In 2009, a multimarker panel (OVA1), which contains CA125, transthyretin, apolipoprotein A1, β2-microglobulin, and transferrin, was cleared by the US Food and Drug Administration (FDA) to identify the likelihood of tumor malignancy, but also not for screening.10 Recently, several promising multiplex biomarker panels have been developed,11 which show improved sensitivity and specificity in detecting ovarian cancer compared to CA125 alone.12−17 However, to achieve higher sensitivity and specificity in detecting ovarian cancer, especially in detecting early stage ovarian cancer, identification of additional candidate biomarkers is needed.

Increasing evidence has indicated that abnormal post-translational modifications are associated with cancer progression and that potential biomarkers may be identified based on their changes in protein modifications rather than changes in
protein abundance. In serum, approximately 50% of proteins are known to be glycosylated. Changes in glycosylation of haptoglobin, α1-proteinase inhibitor, α2-macroglobulin, transferrin, and eosinophil-derived neurotoxin have been reported in ovarian cancer.

In previous work, we identified and confirmed fucosylation changes of corticosteroid-binding globulin, serum amyloid p component, complement factor B, and histidine-rich glycoprotein in ovarian cancer. Using lectin arrays, we also found the serum protein response to the lectin SNA was significantly changed in ovarian cancer. The sialoglycoproteins with aberrant expression in serum of ovarian cancer patients, however, largely remain unidentified. Therefore, the aim of this study was to identify novel sialoglycoprotein biomarkers that could potentially synergize with CA125 for detection of ovarian cancer with improved sensitivity and specificity.

Based on an ExacTag labeling quantitative strategy, five differentially expressed sialoglycoproteins were identified. Using two independent sample sets, we confirmed abnormal sialylation of CLUS, CFH, and HEMO, and altered protein levels of CLUS and LRG1. Finally, CLUS, LRG1, and HEMO were identified as the most promising candidate markers to supplement CA125 for detecting ovarian cancer. The altered sialoglycoproteins discovered by a combination of quantitative proteomics, lectin-based ELISA and ELISA assays provide sialylated glycoproteins as potential biomarkers of ovarian cancer (Figure 1).

**MATERIALS AND METHODS**

**Serum Samples**

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 serum bank is a small discovery set of samples used to identify novel serum markers for ovarian cancer. This set of samples 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. Blood samples were collected by venipuncture directly into serum separator tubes and centrifuged for 12 min at room temperature, and serum aliquots were frozen at −70 °C until use.

A second serum bank (confirmation I) which consisted of 15 healthy controls, 18 benign ovarian diseases, 21 stage I/II ovarian cancers, and 29 stage III ovarian cancers, were used for initial confirmation. All of the healthy controls were provided by the Early Detection Research Network and the Great Lakes-New England CVC (EDRNGNE). In the second serum bank, 8 benign, 21 stage I/II, and 12 stage III ovarian cancer samples were obtained from ProteoGenex (Manhattan Beach, CA), while the other samples were from the first serum bank.

A third serum bank (confirmation II) that was used as an independent confirmation was comprised of 15 healthy controls, 30 benign, 17 stage I/II, and 26 stage III/IV cancer. The 15 healthy controls were from EDRN-GLNE. The 30 benign, 17 stage I/II, and 26 stage III/IV cancer samples were from a sample set provided by the Gynecologic Oncology Group (GOG). A summary of clinical data is given in Table 1.

**Immunodepletion of High Abundance Proteins**

Twelve high abundance serum proteins were removed from 250 μL of each serum sample by a human IgY-12 LC-10 column kit (Beckman Coulter, Brea, CA). The final volume of collected flow-through was concentrated to 500 μL using an YM-3 centrifugal device (Millipore Corp., Bradford, MA). The final protein concentration of each sample was measured using a protein assay kit (Bio-Rad, Hercules, CA).

**ExacTag Labeling and Lectin Extraction of Sialylated Proteins**

ExacTag isobaric reagents (PerkinElmer) were used to label 100 μg of protein from each depleted serum sample as previously described. The labeled samples were mixed together, then the buffer was exchanged to lectin binding buffer (1× PBS, 1 mM MgCl₂, and 1 mM MnCl₂) using Ultracel YM-3.

Agarose-bound SNA, which was selected based on the lectin array experiment, was used to extract sialylated glycoproteins. Columns packed with 1 mL of SNA 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 (0.5 M lactose in PBS followed by 0.5 M lactose in 0.2 M acetic acid). The sample was concentrated using Microcon YM-3 to 200 μL in 25 mM NH₄HCO₃.

**Mass Spectrometry**

The proteins eluted from SNA columns were digested with trypsin at 37 °C overnight, and then PNGase F (New England Biolabs, Ipswich, MA) was used to remove N-Glycans from asparagine (Asn) residues before the resulting peptides were analyzed by LC−MS/MS in a LTQ mass spectrometer (Thermo Finnigan, San Jose, CA). Chromatographic separation of peptides was performed on a Paradigm MG4 micropump system (Michrom Biosciences, Inc., Auburn, CA) equipped with a C18 separation column (0.1 mm × 150 mm, C18 AQ particles, 5 μm, 200 Å, Michrom Biosciences, Inc., Auburn, CA). Peptides were separated with a linear gradient of...
Table 1. Characteristics of the Patients<sup>a</sup>

<table>
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<th>Histology</th>
<th>discovery</th>
<th>confirmation 1</th>
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<td>benign</td>
<td>stage III</td>
</tr>
<tr>
<td></td>
<td>n = 12</td>
<td>n = 22</td>
</tr>
<tr>
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<tr>
<td>Median age (range, year)</td>
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<td>63 (42–78)</td>
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</tr>
<tr>
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<td>n = 15</td>
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</tr>
<tr>
<td></td>
<td>stage I/II</td>
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</tr>
<tr>
<td></td>
<td>stage III</td>
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<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>Histology</td>
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<td>Serous</td>
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</tr>
<tr>
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<td></td>
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<td>13</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>17</td>
</tr>
</tbody>
</table>
| Abbreviation: n/a, not applicable. <sup>b</sup>Fibroids and ovarian thecoma for benign diseases; poorly differentiated adenocarcinoma and fallopian tube carcinoma for ovarian cancer. For some ovarian cancer patients, the grade information was not available.

**ELISA Assay**

The underlying protein abundances of CLUS, CFH, LRG1, HEMO, and VDB were measured by ELISA assay. The CA125 level was also measured in this study. The ELISA kit for CLUS was purchased from R&D Systems (Minneapolis, MN). ELISA kits for HEMO, VDB, and CA125 were purchased from Genway (San Diego, CA). ELISA kit for CFH was from Hycult Biotech (Plymouth Meeting, PA). ELISA kit for LRG1 was purchased from IBL International (Hamburg, Germany). ELISA assays were performed following the manufacturer’s instructions. The absorbance values were read on a microplate reader (BioTek, Synergy HT) at a wavelength of 450 nm.

**Western Blotting Analysis of Candidate Markers after Lectin Enrichment**

Protein extraction using an SNA column was as described above. One hundred microliters of agarose bound SNA was used to process 100 μg of depleted serum proteins. The SNA bound and unbound fractions were both collected, and then separated by SDS-PAGE. Ten micrograms of depleted serum proteins with or without PNGase F cleavage were also loaded. The resolved proteins were transferred onto a PVDF membrane (Bio-Rad). The membrane was blocked by 3% nonfat milk in PBST (0.1% Tween-20 in PBS) for 1 h. The membrane was probed with various primary antibodies at 4 °C overnight. Antibodies used include: mouse antilaminin, mouse antitherminopeptin, mouse anticomplement factor H, and mouse antivitamin D-binding protein. All the antibodies were purchased from Abcam (Cambridge, MA).

**Statistical Analysis**

All statistical analyses were performed using SPSS 11.5. Statistical differences were determined using the Student’s t test, one-way analysis of variance (ANOVA), or Wilcoxon rank-sum test. 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 the ELISA and Western blotting results.
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 AND DISCUSSION

#### Enrichment of Glycoproteins by Lectin Affinity Chromatography

Glycosylation is an important post-translational modification and has been reported to play an important role in cancer progression. A number of serum glycoproteins have been used as diagnostic or prognostic biomarkers for a variety of cancers. In our previous work, we found higher expression of sialylated glycoproteins in the serum of ovarian cancer patients compared with benign diseases by lectin array. Therefore, we used SNA, a sialic acid specific lectin, to capture sialylated glycoproteins in this work. Through lectin-blots, we verified that sialylated glycoproteins were specifically enriched by SNA as shown in Supplemental Figure S1, Supporting Information.

#### Differentially Expressed Sialylglycoproteins

To identify differentially expressed glycoproteins in the serum of ovarian cancer patients, immunodepleted serum proteins were labeled with ExacTag isobaric tags and extracted using a lectin column. On the basis of the lectin-array results from our previous studies, SNA was used to extract the sialylated proteins. After quantitative LC–MS/MS analysis, CLUS, HEMO, VDB, LRG1 and CFH, were identified as displaying abnormal expression levels in ovarian cancer. Detailed information on these proteins is listed in Table 2.

#### Evaluation of Analytical Performance of Lectin-based ELISA Assay

In order to analyze glycosylation changes of proteins, an antibody microarray strategy was developed by Chen et al. To prevent nonspecific binding of the antibodies to lectins, in the antibody microarray, the glycans on the antibodies were oxidized by sodium periodate (NaIO₄) followed by derivatization with MPBH and dipeptide solution (Cys-Gly). Unfortunately, periodate oxidation can diminish or inactivate antibody immunoreactivities. This may increase nonspecific bindings leading to inaccurate quantitative results. In this study, the immunoreactivity and specificity of anti-VDB were found to be decreased after the oxidation with NaIO₄ (data not shown). Furthermore, precipitates are formed during the process of oxidation with NaIO₄ which can increase background if not completely removed. Therefore, the oxidation and derivatization process was found not to be optimal for lectin-based ELISA assays for this study.

Herein, we developed a lectin-based ELISA to achieve the expression analysis of a specific sialylated glycoprotein from crude serum samples without the use of the oxidation and derivatization process (Figure 2a). The concentration of lectins coated on the 96-well plate was optimized to achieve a low background. Biotin/HRP-conjugated primary antibodies were then used to detect the specific glycoproteins from the serum samples. The result showed a high ratio of signal to background (more than 6) was obtained with this procedure (Figure 2b).

As shown in Figure 2b, the standard curves for the glycoproteins CLUS, CFH, and HEMO were within the linear range (Figure 2b). The absorbance values measured at 450 nm.

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**Table 2. Significantly Changed Proteins between Stage III Ovarian Cancer and Benign Diseases Detected by ExacTag Labeling-based Quantitative LC–MS/MS Analysis after SNA Enrichment**

<table>
<thead>
<tr>
<th>protein ID</th>
<th>protein name</th>
<th>protein description</th>
<th>P-value</th>
<th>function</th>
</tr>
</thead>
<tbody>
<tr>
<td>P08603</td>
<td>CFH</td>
<td>Complement factor H</td>
<td>0.015</td>
<td>Part of complement system</td>
</tr>
<tr>
<td>P02750</td>
<td>LRG1</td>
<td>Leucine-rich alpha-2-glycoprotein</td>
<td>0.019</td>
<td>It is expressed during granulocyte differentiation</td>
</tr>
<tr>
<td>P02774</td>
<td>VDB</td>
<td>Vitamin D-binding protein</td>
<td>0.024</td>
<td>In plasma, it carries the vitamin D sterols and prevents polymerization of actin by binding its monomers.</td>
</tr>
<tr>
<td>P02790</td>
<td>HEMO</td>
<td>Hemopexin</td>
<td>0.039</td>
<td>Heme transporter activity</td>
</tr>
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<td>P10909</td>
<td>CLUS</td>
<td>Clusterin</td>
<td>0.046</td>
<td>Prevents stress-induced aggregation of blood plasma proteins.</td>
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*P-value: statistical significance of changed proteins between ovarian cancer and benign diseases after Student’s t-test.*

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**Figure 2.** Detection of glycosylation patterns using lectin-based ELISA assay. (a) Diagram of lectin-based ELISA assay for the analysis of target glycoproteins. (b) Response curves of sialylated CLUS, HEMO, CFH, and VDB to different dilutions of serum. The values on the x-axis are the dilution factors of the serum before being hybridized with the antibodies. The y-axis is the absorbance values measured at 450 nm.
range between a dilution of 50-fold and 800-fold, while the intensities for VDB were lower and showed no obvious changes over different dilutions of serum samples. As SNA prefers to bind N-linked glycoproteins that contain NeuNac2–6 residues, the low intensities for VDB may be due to a low sialylation level or lack of N-glycans present. We then enriched the sialylated glycoproteins with SNA, followed by Western blotting assay to verify the VDB results. As shown in Figure 3, a weak band of VDB was detected in the elution fraction, while a strong band appeared in the flow-through fraction, which indicated the low affinity of VDB to SNA. Furthermore, before Western blotting analysis, serum samples were treated with PNGase F to determine whether N-glycans were attached to VDB. As shown in Figure 3, the molecular weight of VDB showed no change after treatment with PNGase F, suggesting that there were no N-glycans attached to VDB.

The glycosylation status of VDB in serum samples has been reported in several previous studies.31–33 VDB contains an N-glycosylation consensus sequence and the potential N-glycosylation of VDB has been reported in the UniProtKB/Swiss-Prot database and a report by Wang et al.31 However, with more than 100 serum samples analyzed by mass spectrometry, Borges et al.32 identified O-glycosylation of VDB with a trisaccharide of NeuNAc-Gal-GalNAc attached and did not find N-glycosylation of VDB. Their results are consistent with the findings of Viau et al.33 In the present study, we confirmed that VDB is a non-N-linked glycoprotein with low affinity to SNA.

**Confirmation of Expression Change of Sialylated Glycoproteins by Lectin-based ELISA Assay**

From the mass spectrometry analysis, we identified five candidate biomarkers, CLUS, CFH, HEMO, VDB, and LRG1 with abnormal sialylation levels which could be used for detection of ovarian cancer. An SNA-based ELISA assay was used to confirm the sialylation changes of these candidates from the crude serum samples without depletion of high abundance proteins. As described above, the lectin-based ELISA assay requires a biotin or HRP-conjugated primary antibody. However, for LRG1, there was no biotin or HRP-conjugated antibody available. As a result, the sialylation levels of LRG1 were not measured in this study. CA125 is a low abundance (ng/mL range), high molecular weight glycoprotein (>200 kDa) in serum. It has been reported that no more than one

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**Figure 3.** Western blotting analysis of CFH, HEMO, CLUS, and VDB before/after SNA enrichment. Lane 1, 10 μg serum proteins with N-glycans removed by PNGase F; lane 2, 10 μg serum proteins without PNGase F treatment; lane 3, the flow-through unbound fraction of SNA enrichment assay; lane 4, the elution bound fraction of SNA enrichment.

**Figure 4.** 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.
Sialic acid is attached to the N-glycans of CA125 and the N-glycosylation on CA125 does not contribute to the major changes in glycan levels in sera from ovarian patients. Therefore, sialylation levels of CA125 were not analyzed in this study.

Through the SNA-based ELISA assay, we found, consistent with the LC−MS/MS quantification, that CLUS, HEMO, and CFH showed significantly lower sialylation levels in stage III cancer sera than in the benign sera (p < 0.05, Figure 4a). A ROC curve was constructed for each of the three changed sialylated proteins to differentiate stage III cancer from benign diseases as shown in Figure 4b. The AUC for sialylated CLUS, CFH, and HEMO was 0.704, 0.719, and 0.708, respectively.

Besides benign diseases and patients with stage III ovarian cancer, we also measured the abundance of sialylated CLUS, HEMO, and CFH in healthy controls and patients with early stage (I/II) cancer using SNA-based ELISA assay. Compared to healthy controls, the sialylation of all of the above three proteins were significantly decreased in stage III cancer samples (p < 0.05, Figure 4a). The AUC for sialylated CLUS, CFH, and HEMO to distinguish stage III cancer from healthy controls was 0.883, 0.799, and 0.784, respectively (Figure 4b). We further found that sialylated CLUS, CFH, and HEMO were decreased in early stage ovarian cancer compared to healthy controls. The AUC for sialylated CLUS, CFH, and HEMO to differentiate early stage cancer from healthy controls was 0.733, 0.686, and 0.741 (Figure 4b).

An independent set of samples from GOG (confirmation II) was used to verify the observed sialylation changes. We confirmed the decreased sialylation of CLUS, CFH, and HEMO in stage III cancer compared to benign diseases and healthy controls (Supplemental Figure S2, Supporting Information). Importantly, we verified the decreased sialylation of CLUS, HEMO, and CFH in early stage ovarian cancer as shown in Supplemental Figure S2, Supporting Information.

Since sialic acids are located at the terminal positions of glycans attached to the glycoproteins, sialylation determines the half-lives of many circulating glycoproteins and plays critical roles in cell−cell interactions, cell adhesion, and protein targeting. Altered sialylation has been found to be involved in critical pathological events during cancer progression, including cancer transformation and metastasis. Abnormal expressions of sialylated glycoproteins have been discovered in many cancers such as pancreatic, colon, brain, and breast.

Figure 5. Underlying protein levels of individual candidate biomarkers measured by ELISA kits. (a) 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.
The deregulation of sialyltransferase enzymatic activity has been reported to account for the abnormal expression of sialylation in tumors, but the precise mechanisms remain unknown. Altered mRNA expression of sialyltransferases has been found in malignant ovarian cancers; however, there are few reports related to identification of expression changes of sialoglycoproteins associated with ovarian cancer. In this study, by applying an integrated platform using the lectin array and quantitative glycoproteomics analysis, differentially expressed sialylated glycoproteins were identified from the sera of ovarian cancer patients. Furthermore, using the SNA-based ELISA assay described herein, the altered expression of sialylated glycoproteins was verified using two independent sample sets. From our results, we can conclude that sialylation changes are associated with ovarian cancer progression and altered protein sialylations could be potentially used to detect ovarian cancer.

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

Since the abundance changes of the underlying protein could account for the detected glycosylation changes, an ELISA assay was used to measure the underlying protein concentrations of CLUS, LRG1, CFH, HEMO, and VDB from the original serum samples using the initial confirmation sample set (confirmation I). Among the five proteins measured by ELISA, VDB, HEMO, and CFH showed no statistically significant changes between ovarian cancer and healthy controls or benign diseases (p > 0.05 for all pairwise comparisons) using the initial confirmation sample set. The total abundance of CLUS consistently decreased from healthy controls to stage III cancer, while the protein levels of LRG1 were increased in cancer (Figure 5a). CA125 level was significantly increased in the serum of ovarian cancer patients as shown in Supplemental Figure S3, Supporting Information.

ROC curves for CLUS and LRG1 were constructed to differentiate stage III ovarian cancer from healthy controls (Figure 5b). The AUC for CLUS and LRG1 were 0.86 and 0.83, respectively. CA125 achieved the highest AUC (0.922) for differentiation of stage III ovarian cancer from healthy controls. However, the combination of CLUS and LRG1 had an AUC of 0.954 with a specificity of 100% at a sensitivity of 90%, which outperforms CA125 alone.

Since benign diseases may cause elevation of CA125 levels, it is essential to identify complementary markers to supplement CA125 in order to improve its performance in differentiating ovarian cancer from benign diseases. After statistical analysis, CLUS and LRG1 levels were found to be significantly changed between stage III cancer and benign diseases. The concentrations of CLUS decreased from a mean of 394.3 μg/mL (range, 181.4–974.5 μg/mL) in benign diseases to 230.8 μg/mL (range, 101.8–395.8 μg/mL) in stage III ovarian cancer, while LRG1 concentrations increased from a mean of 13.28 μg/mL (range, 4.12–40.72 μg/mL) in benign diseases to 25.69 μg/mL (range, 6.8–63.25 μg/mL) in stage III cancer (p < 0.05). ROC curves for CLUS and LRG1 were constructed to differentiate stage III ovarian cancer from benign diseases (Figure 5b). The AUC for CLUS and LRG1 were 0.768 and 0.767, respectively. The AUC for CA125 was 0.811 for differentiation of stage III ovarian cancer from benign diseases. The combination of CLUS and LRG1 had an AUC of 0.837 with a specificity of 89% at a sensitivity of 66%, which outperforms CA125 to differentiate stage III ovarian cancer from benign diseases. Further, the combination of CLUS, LRG1, and CA125 resulted in an AUC of 0.872 with a specificity of 94% at a sensitivity of 66%, which improved both sensitivity and specificity when compared to CA125 alone.

Due to the lack of symptoms in early stage ovarian cancer, it is important to identify candidate biomarkers to detect cancer in early stage in order to reduce the mortality. In this study, we found LRG1 was significantly increased in early stage ovarian cancer. The AUC value for LRG1 was 0.743 for distinguishing early stage ovarian cancer from healthy controls, while CA125
Figure 7. Western blotting analysis of protein levels of CFH and CLUS. Depleted serum samples from 6 ovarian cancer and 6 benign diseases were analyzed respectively. Ten micograms of protein were separated by a 4-12.5% gel. The expression level of CLUS was decreased in the serum from ovarian cancer patients, while CFH showed no significant difference between ovarian cancer and benign diseases.

had an AUC of 0.816 (Figure 5b). The combination of LRG1 and CA125 had an AUC of 0.863 with a specificity of 87% at a sensitivity of 81% for detecting early stage ovarian cancer. The LRG1 level was also found to be significantly increased in early stage ovarian cancer compared to benign diseases. The AUC for LRG1 and CA125 was 0.685 and 0.687, respectively, as shown in Figure 5b.

Ultimately, we used an independent serum bank from GOG (confirmation II) to verify the protein level changes of the five candidate markers identified by LC−MS/MS. We confirmed that the protein level of CLUS was decreased in ovarian cancer compared to benign diseases or healthy controls, while LRG1 was increased in ovarian cancer (Figure 6a). We confirmed that the combination of LRG1 and CLUS had an AUC of 0.854, which showed comparable performance to CA125 to differentiate late stage ovarian cancer from benign diseases (Figure 6b). Importantly, we verified the performance of LRG1 and CLUS in distinguishing early stage ovarian cancer from healthy controls and also found protein levels of HEMO were decreased in early stage ovarian cancer (Figure 6a). The combination of CLUS, LRG and HEMO resulted in an AUC of 0.894 with a specificity of 93% at a sensitivity of 82% for detecting early stage ovarian cancer, which outperforms the CA125 alone (AUC = 0.725) as shown in Figure 6b.

Human LRG1 is a serum glycoprotein of 347 amino acids in length, with five potential glycosylation sites.44 Elevated levels of LRG1 have been found in a variety of cancers, including pancreatic cancer,45 lung cancer,46 and hepatocellular carcinoma.47 LRG1 is produced by the liver in response to infection or injury, and has been classified as an acute-phase protein. Due to its binding to cytochrome c, LRG1 has also been reported to play an important role in cell survival and apoptosis. Furthermore, Andersen et al.48 found that ovarian cancer cells could secrete LRG1 and suggested the direct relationship between tumor burden and serum levels of LRG1. In this study, using two independent sample sets, we verified the increased level of LRG1 in ovarian cancer, especially in early stage ovarian cancer. Our results indicated that LRG1 could be used as a promising candidate marker to detect ovarian cancer from benign diseases or healthy controls.

Clusterin, also known as Apolipoprotein J (ApoJ), is a highly conserved, secreted heterodimeric glycoprotein, with seven potential glycosylation sites.49 CLUS has been implicated in diverse physiological processes, including cell adhesion, cell cycle regulation, apoptotic cell death, tissue remodeling, and immune system regulation,50,51 most of which are involved in carcinogenesis and tumor growth. Increased CLUS levels have been described in breast,52 colon,53 lung,54 and prostatic carcinoma.55 However, decreased CLUS levels were also reported in a number of cancers, including esophageal squamous cell carcinoma,56 pancreatic,57 and prostatic cancers.58 Accumulating evidence indicates that different isoforms of CLUS exist in tissues and human fluids, and that these different isoforms exhibit their own specific functions in tumor progression, which may explain the disparity observed in the different studies.59 Two CLUS isoforms are generated in human cells, the secretory form (sCLUS) and the nuclear form (nCLUS). The nCLUS is expressed in the cytoplasm and nucleus. In response to cell damage, the cytoplasmic CLUS, which functions as a cytoprotective molecule, may translocate from cytoplasm to the nucleus where the nCLUS is accumulated and leads to cell death.60

Protein level changes of CLUS have been found in ovarian cancer tissues and ovarian cancer cell lines.51,60 However, there are few studies regarding alteration of CLUS in serum. In this study, CLUS levels were evaluated by Western blotting analysis and were found to exist as a full-length uncleaved ~60 kDa protein in serum, which is also highly N-glycosylated where a ~40 kDa band was detected after PNGase F cleavage (Figure 3). Using two independent sample sets, we verified the decreased CLUS levels in sera of ovarian cancer patients. We also found CLUS showed a good performance in distinguishing stage III ovarian cancer from healthy controls (AUC=0.86) or benign diseases (AUC = 0.768). The expression changes of CLUS between ovarian cancer and benign diseases were further verified by Western blotting analysis as shown in Figure 7.

With the initial confirmation sample set, the protein levels of CFH and HEMO showed no significant changes in the sera of patients with ovarian cancer, while their sialylation levels were confirmed to be significantly changed using two independent sample sets. From the results, we can conclude that sialylation changes and protein level changes could be complementary to each other and potentially used to detect ovarian cancer.

**CONCLUSIONS**

We have applied a comprehensive strategy for identification and confirmation of sialylated glycoprotein biomarkers for the detection of ovarian cancer. Using a lectin-array, we found aberrant sialylation levels in serum glycoproteins from patients with ovarian cancer. We found that sialylated CLUS, LRG1, CFH, VDB, and HEMO contributed to the sialylation changes detected by the lectin-array. We then employed an SNA-based ELISA assay to verify these changes. The abnormal expression of sialylated CLUS, CFH, and HEMO was further verified by SNA-based ELISA assay.

In order to clarify whether sialylation changes were from the underlying protein abundance changes or actual changes of glycosylation, we further measured the protein concentrations
by ELISA assay. The protein abundance of CLUS was found to be significantly decreased in sera of ovarian cancer patients, while protein levels of LRG1 were increased in cancer patient serum.

Finally, an independent sample set was used to confirm the decreased sialylation level of CLUS, CFH and HEMO in ovarian cancer, as well as the enhanced performance of CLUS, LRG1 and HEMO in differentiating early stage ovarian cancer from healthy controls. Our work provides a comprehensive strategy to identify novel or complementary glycoprotein biomarkers to supplement CA125 for detection of ovarian cancer with improved sensitivity and specificity. The performance of these candidate markers in detecting ovarian cancer will be further validated in a large number of serum samples.

ASSOCIATED CONTENT

S Supporting Information

The Exactag labeled samples are listed in Supplemental Table S1. Proteins identified from the SNA fractions are listed in Supplemental Table S2. Sialylated proteins detected by lectin-blot are shown in Supplemental Figure S1. The ROC curves for sialylated CLUS, LRG1, and HEMO to distinguish ovarian cancer from benign diseases or healthy controls by using confirmation sample set II are shown in Supplemental Figure S2. The CA125 levels measured from confirmation sample set I and confirmation sample set II are shown in Supplemental Figure S3. 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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Analysis of Glycan Variation on Glycoproteins from Serum by the Reverse Lectin-Based ELISA Assay

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ABSTRACT: Altered glycosylation in glycoproteins is associated with carcinogenesis, and certain glycan structures and glycoproteins are well-known markers for tumor progression. To identify potential diagnostic candidate markers, we have developed a novel method for analysis of glycosylation changes of glycoproteins from crude serum samples using lectin-based glycoprotein capture followed by detection with biotin/HRP-conjugated antibodies. The amount of lectin coated on the microplate well was optimized to achieve low background and improved S/N compared with current lectin ELISA methods. In the presence of competing sugars of lectin AAL or with sialic acid removed from the glycoproteins, we confirmed that this method specifically detects glycosylation changes of proteins rather than protein abundance variation. Using our reverse lectin-based ELISA assay, increased fucosylated haptoglobin was observed in sera of patients with ovarian cancer, while the protein level of haptoglobin remained the same between cancers and noncases. The combination of fucosylated haptoglobin and CA125 (AUC = 0.88) showed improved performance for distinguishing stage-III ovarian cancer from noncases compared with CA125 alone (AUC = 0.86). In differentiating early-stage ovarian cancer from noncases, fucosylated haptoglobin showed comparable performance to CA125. The combination of CA125 and fucosylated haptoglobin resulted in an AUC of 0.855, which outperforms CA125 to distinguish early-stage cancer from noncases. Our study provides an alternative method to quantify glycosylation changes of proteins from serum samples, which will be essential for biomarker discovery and validation studies.

KEYWORDS: reverse lectin-based ELISA, glycosylation, biomarkers, ovarian cancer

INTRODUCTION

Glycosylation is a posttranslational modification that has significantly contributed to protein–protein interactions, cellular recognition, and, in particular, cancer development and progression.1–3 Increasing evidence has indicated that abnormal posttranslational modifications are associated with cancer progression and that potential biomarkers may be identified based on their changes in protein modifications.4–6 Glycosylation changes in serum proteins have been reported to contribute to the progression of various cancers, including pancreatic,5 ovarian,6 hepatocellular,7 and breast cancer.8 Therefore, analysis of glycosylation changes of serum glycoproteins may provide a promising strategy to identify new diagnostic biomarkers.

Methods to identify and quantify variations in glycosylation in complex biological samples have been widely established, which are mainly based on the removal of glycans by enzymatic digestion, followed by chromatographic separation and mass spectrometry analysis.9,10 These methods can provide detailed information about glycan structures but are not suitable for analyzing a large number of biological samples. Because of the lack of glycopeptide standards, SRM/MRM assays, which have been widely used to quantify protein level changes, are not optimal for quantifying glycosylation changes of target proteins. Because a number of glycosylated proteins in serum have been identified as promising candidate biomarkers, an improved method for quantification of glycosylation changes of proteins from original sera is needed.

Recently, lectin-based antibody microarrays and lectin-ELISA assays have been developed to analyze glycosylation changes of proteins from serum samples.11–13 The lectin-based antibody microarrays and the lectin-ELISA assay are both based on coating antibodies to the slides or 96-well plates, where the glycans on the antibodies need to be oxidized by sodium periodate (NaIO4), followed by derivatization with MPBH and dipeptide solution (Cys-Gly). Unfortunately, periodate oxidation can diminish or inactivate antibody immunoreactivities,14 which may increase nonspecific binding and lead to inaccurate quantitative results. Furthermore, precipitates are formed during the process of oxidation with NaIO4 which can increase background if not completely removed.15

Herein, we have developed an alternative method, a reverse lectin-based ELISA assay to quantify glycosylated proteins from crude serum samples without the oxidation and derivatization of glycans on the antibodies. Because fucosylation/sialylation
Developing Reverse Lectin-Based ELISA Assay

A reverse lectin-based ELISA assay was developed to analyze changes in glycosylation changes in proteins from crude serum samples based on the differential binding of glycoproteins to their specific lectins. One hundred micrograms of lectin (1.2S, 2.5, S, 10, 50, 100 μg/mL AAL or 1, S, 10, 50 μg/mL SNA) was added to each well of a 96-well ELISA plate (Thermo Scientific, IL) and incubated at 37 °C for 2 h. After the lectin solution was removed, the plate was washed five times with PBST (0.1% Tween-20 in PBS). The plate was then blocked with 3% BSA in PBST for 1 h. One hundred microliters of each 200-fold diluted serum sample or 2 μg of purified protein was applied to each well of a 96-well ELISA plate. After 1 h of incubation, the plate was washed with PBST five times to remove unbound proteins. One hundred microliters of biotinylated/HRP-conjugated antibodies (1:1000 for antihaptoglobin, and 1:50,000 for anti-IgG) purchased from Abcam (Cambridge, MA) was added to bind with their corresponding antigens. TMB working solution was added to each well, followed by stop solution. The absorbance values were read on a microplate reader (BioTek, Synergy HT) at a wavelength of 450 nm.

**Lectin-Blot for Haptoglobin and IgG**

Human full-length proteins of haptoglobin and IgG were purchased from Abcam (Cambridge, MA). Five micrograms of the proteins was separated by SDS-PAGE. The resolved proteins were then transferred onto a PVDF membrane (Bio-Rad). The membrane was blocked by 3% nonfat milk in PBST (0.1% Tween-20 in PBS) for 1 h. The membrane was probed with 0.5 μg/mL biotinylated AAL or biotinylated SNA to bind with their preferred oligosaccharides. HRP-conjugated streptavidin (1 μg/mL) was then added, and the blot was detected by DAB detection kits.

**ELISA Assay**

The protein abundances of HAP, IgG, and CA125 in sera of ovarian cancer and control groups were measured by ELISA assay. ELISA kits for HAP, IgG, and CA125 were all purchased from Genway (San Diego, CA).

**Statistical Analysis**

All statistical analyses were performed using SPSS 11.5. Statistical differences were determined using Wilcoxon rank-sum test. 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 AND DISCUSSION**

**Determining the Presence of Fucosylation/Sialylation of Haptoglobin and IgG by Lectin-Blot**

Using the reverse lectin-based ELISA assay, we found high responses of haptoglobin and IgG to AAL or SNA. To exclude false-positives, we used lectin-blots to verify the presence of fucosylated/sialylated glycans on haptoglobin and IgG. AAL is a lectin that responds to fucose linked (α1-2,6 or α1-2,3 linkage) to N-acetyllactosamine or to fucose linked (α1-3) to N-acetyllactosamine related structures, while SNA responds to sialic acid attached to terminal galactose in α2-6 or α2-3 linkage. As shown in Supplemental Figure S1 in the Supporting Information, the reverse lectin-based ELISA assay to quantify fucosylation/sialylation changes of target proteins in ovarian cancer. The reverse lectin-based ELISA assay is based on coating lectin on the microtiter plate followed by detection of captured glycoproteins with biotin/HRP-conjugated primary antibodies. The concentration of lectins coated on the 96-well plate was optimized to achieve a low background compared with the current lectin-ELISA format. With this method, we confirmed the increased expression of fucosylated haptoglobin in ovarian cancer, especially in early-stage ovarian cancer. Our findings suggest that fucosylated haptoglobin could be a promising candidate biomarker, which can supplement the clinically used biomarker CA125 (cancer antigen 125) to detect ovarian cancer with improved sensitivity and specificity.

**Table 1. Characteristics of the Patients**

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Abbreviation: n/a, not applicable.


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Technical Note

The presence of fucosylated/sialylated glycans on the haptoglobin and IgG, which had responses to AAL/SNA detection.

Optimizing Reverse Lectin-Based ELISA Assay Conditions

The workflow of the reverse lectin-based ELISA assay is shown in Figure 1. In this experiment, a number of control determinations with the removal of serum or lectin from the mixture of reverse lectin-based ELISA assay were tested to avoid potential interfering factors. To reduce the background caused by nonspecific binding of serum proteins or biotinylated/HRP-conjugated antibodies to the 96-well plate, a buffer containing 1% BSA is used for the dilution of the serum and antibodies. In addition, we also measured the absorbance values when lectin, serum, or antibody was omitted from the reverse lectin-based ELISA assay. To measure sialylated haptoglobin by reverse SNA-based ELISA assay, low absorbance values were obtained when lectin or serum was removed from the assay. The results indicate that there was no nonspecific binding of serum proteins to the plate, and the glycans on the antibodies had no effect on detection of the sialylated haptoglobin. To measure fucosylated haptoglobin by reverse AAL-based ELISA assay, low absorbance values were obtained when lectin was omitted from the assay, while a slightly higher absorbance value was obtained when serum was left out of the assay, indicating the presence of fucosylated glycans on the antibody. The S/N ratio of the reverse AAL-based ELISA assay for detecting fucosylated haptoglobin is still higher than 3, as shown in Supplemental Table S1 in the Supporting Information. Therefore, the low binding of AAL to the glycans on the antibodies did not influence the quantification of fucosylated haptoglobin from the serum samples.

Because of the high abundance of IgG in the serum, we observed absorbance values when lectin was left out of the reverse SNA/AAL-based ELISA assay. However, compared with the high absorbance values for the sample group, this low background did not influence the accuracy of quantifying fucosylated or sialylated IgG from crude serum samples.

For detection of fucosylated IgG by reverse AAL-based ELISA assay, high background was observed when serum was omitted from the assay, indicating high binding of AAL to the fucosylated glycans on the anti-IgG (Fc). Attempts to reduce this background by oxidizing the glycans on the anti-IgG (Fc) were unsuccessful. Finally, we found that using anti-IgG (Fab) in place of anti-IgG (Fc) could dramatically reduce the background caused by the binding of AAL to fucosylated glycans on the antibodies. The high background obtained by anti-IgG (Fc) is due to the presence of fucosylated oligosaccharide in its Fc portion (Supplemental Table S1 in the Supporting Information). Antibodies with the F(ab) portion have been used in several studies to eliminate the binding of lectin to glycans on the antibodies.18,19

Optimizing Concentration of Lectins for Well-Coating

To reduce the background and increase the S/N ratio of the assay, we further optimized the amount of lectin for well coating. The data on optimization of AAL concentration used for well coating are shown in Figure 2. When the amount of AAL for well coating increased, higher intensities for both test and control (no serum) group were obtained (Figure 2a). The highest S/N ratio (＞5) was obtained when 5 μg/mL AAL was used for coating the plate (Figure 2b). Therefore, 5 μg/mL AAL was chosen for the well coating for the reverse AAL-based ELISA assay.

Compared with reverse AAL-based ELISA assay, lower background was obtained for the reverse SNA-based ELISA assay (Figure 2c), which may be due to the lower sialylation level of the tested antibodies. The S/N ratio reached the maximum when 10 μg/mL SNA was applied for well coating, and the higher concentration of SNA (50 μg/mL) did not increase the amount of bound protein. Therefore, 10 μg/mL SNA was used for well coating for reverse SNA-based ELISA assay, as shown in Figure 2d.

Evaluating the Specificity of the Reverse Lectin-Based ELISA Assay

To verify that AAL specifically bound to fucosylated glycans on the glycoproteins rather than nonspecifically interacted with other glycan structures, we measured AAL binding performance in the presence of competing sugars, l-fucose. As shown in Figure 3a, preincubation of AAL with competing sugar, l-fucose, resulted in a dramatic reduction in AAL binding to fucosylated haptoglobin after serum/protein incubation. A parallel experiment using lactose showed no effect on AAL binding performance. The reduced binding of AAL to fucosylated proteins using its competing sugar indicates the specific binding of AAL to fucosylated glycans on the captured proteins.

To study the specificity of the reverse SNA-based ELISA assay, we tested the lectin binding to the captured proteins by treating samples with neuraminidase to remove the sialic acid of the glycoproteins. After samples were treated with neuraminidase, no proteins were found to bind to SNA when detected by antihaptoglobin (HRP), while serum/protein without sialic acid removal showed the high binding to SNA, as shown in Figure 3b. The results confirmed that lectins specifically bind to glycans rather than nonspecifically interact with proteins. The specificity of reverse AAL/SNA-based ELISA assays were also confirmed by testing protein IgG (Supplemental Figure S2 in the Supporting Information). Our results indicated that the reverse lectin-based ELISA assay showed the specific glycosylation changes of proteins.

To further address the specificities of the reverse lectin-based ELISA assay, we tested the specificity of the antibodies of
haptoglobin and IgG used in this study. Serum samples with haptoglobin- and IgG-depleted were used for the reverse lectin-based ELISA assay to check whether these two proteins can still be detected with their antibodies. As shown in Supplemental Figure S3 in the Supporting Information, a significant reduction in antibody bindings to their antigens was observed, which indicated the specificity of the antibodies. The results further confirmed the specificity of the reverse lectin-based ELISA assay to quantify the fucosylation/sialylation changes of haptoglobin and IgG from crude serum samples.

**Reproducibility and Precision of the Reverse Lectin-Based ELISA Assay**

To compare samples analyzed on different microtiter plates or in different laboratories, standard samples need to be used to normalize the results. Because most of standard samples of differentially fucosylated/sialylated proteins are not commercially available, purified proteins were used to establish the standard curves in this study. As shown in Figure 3, haptoglobin showed a linear response in SNA/AAL binding between 5 and 80 ng/mL \( (R^2 = 0.991) \). Although this type of standard curve is not ideal to calculate the absolute concentrations of the glycosylated proteins, it could be successfully used to enable comparisons of samples analyzed on different microtiter plates or in different laboratories.

The intra- and interassay precision of the reverse lectin-based ELISA assay was determined by repeated analysis of the serum samples on the same (in triplicate) and different microtiter plates (in duplicate). The coefficient of variation (CV) for intra- and interassay precision was 3.6 and 5.7%, respectively.

**Determining Glycosylation Changes on Serum Proteins in Cancer Patients**

One of the most important applications of the developed reverse lectin-based ELISA assays is to measure glycosylation changes on serum proteins from a large cohort of patients. The fucosylation/sialylation changes of haptoglobin and IgG in sera of patients with ovarian cancer were measured in duplicate by reverse lectin-based ELISA assay. We found the increased fucosylation levels of haptoglobin in ovarian cancer, especially in early-stage cancer compared with healthy controls or benign diseases (Figure 4), while the sialylated haptoglobin and IgG as well as fucosylated IgG showed no significant changes (Supplemental Figure S4 in the Supporting Information). ROC curves were constructed for the changes in fucosylated glycoproteins to distinguish cases (late stage and early stage cancers) from noncases (healthy controls and benign diseases). The clinically used marker CA125 obtained the highest AUC (0.86) to differentiate cancer from noncases. The AUC for fucosylated haptoglobin was 0.739 (Figure 5). The combination
of CA125 and fucosylated haptoglobin had an AUC of 0.88 with specificity of 96.3% at a sensitivity of 78%, which improved both sensitivity and specificity when compared with CA125 alone (Figure 5). It should be noted that the fucosylated haptoglobin had an AUC of 0.741 to distinguish early stage from noncases, which was comparable to CA125 (0.795). The combination of CA125 and fucosylated haptoglobin resulted in an AUC of 0.855, which outperforms CA125 to distinguish early-stage cancer from noncases (Figure 5).

We performed a power analysis to determine the power of our experiments. At the given sample size, the variance of expression values, and the difference we want to detect (two-tailed, 0.05), the power of the experiment was calculated. The powers at the calculated differences of the means (delta mean) of comparison groups of differentially expressed fucosylated haptoglobin and CA125 are higher than 99%, which provides the statistical support for the number of samples included in our study.

Haptoglobin, a glycosylated protein, is mainly produced in the liver and composed of two α and two β subunits. Four N-linked glycans are attached to each β subunit. Increased fucosylated haptoglobin has also been observed in various types of cancers, such as pancreatic cancer, hepatoma, prostate cancer, lung cancer, and ovarian cancer. There are several key advantages of the reverse lectin-based ELISA method compared with other methods. In these previous studies, to quantify fucosylated haptoglobin, several high-abundance proteins such as IgG were depleted, or haptoglobin needed to be purified from serum samples before mass spectrometry or lectin blotting analysis. Also, a large quantity of purified glycoprotein (micrograms to milligrams) is required for glycan analysis using mass spectrometry, which needs at least 10 μL of sera, while for the reverse lectin-based ELISA assay, nanogram levels of protein or <0.5 μL serum is sufficient to analyze the glycosylation changes of protein. Because glycans need to be released from glycoprotein purified from depleted serum samples before MS analysis, contaminants from other glycoproteins may interfere with glycan quantification of target proteins. In contrast, our results showed high specificity of reverse lectin-based ELISA assay for analyzing the glycosylation changes of target proteins (Figure 3 and Supplemental Figure S3 in the Supporting Information).

Recently, using glycopeptides CID MS/MS and glycan database search, Chandler et al. have studied site-specific N-glycosylation microheterogeneity of haptoglobin, which provided detailed glycosylation patterns of haptoglobin. By site-specific

Figure 3. Determining the specificity of the reverse lectin-based ELISA assay. (a) For reverse AAL-based ELISA assay, OD values were measured after the lectin coated on the microtiter plates had or had not been preincubated with 100-fold molar excess of a competing sugar. (b) For reverse SNA-based ELISA assay, OD values were measured with removal of the sialic acid on the glycoproteins.
specific glycan analysis with LC–ESI–MS, Nakano et al.28 have shown that fucosylated glycans are markedly increased at N211 in pancreatic cancer. However, these studies analyzed haptoglobin glycans in a qualitative instead of a quantitative manner, which are not applicable to quantify glycosylation changes of haptoglobin from individual samples. Quantification methods such as MS and lectin blotting lack sensitivity, accuracy and high sample throughput,29 which may impede their application in clinical examination. A system that is suitable for analyzing a large number of specimens is required. In this study, the developed reverse lectin-based ELISA assay provides a reproducible method to quantify glycosylation changes of proteins from crude serum samples. Because biotinylated/HRP-conjugated antibodies are required for the reverse lectin-based ELISA assay, the glycoproteins that lack commercial biotinylated/HRP-conjugated antibodies could not be analyzed by the reverse lectin-based ELISA assay.

Because the abundance changes of the underlying protein could account for the detected glycosylation changes, an ELISA assay was used to measure the underlying protein concentrations of haptoglobin from the original serum samples. As shown in Figure 4, fucosylated haptoglobin showed significant changes between cancer and noncases, while its protein levels showed no significant changes between cancer and control groups. The results support previous findings that potential

Figure 4. Levels of CA125, haptoglobin, and fucosylated haptoglobin in sera of patients with ovarian cancer were determined by ELISA and reverse lectin-based ELISA assays. CA125 and fucosylated haptoglobin showed significant changes between noncases (normal and benign) and cases (early-stage and late-stage ovarian cancer) (p < 0.01).

Figure 5. ROC analyses for CA125 and fucosylated haptoglobin to differentiate ovarian cancer from noncases.
biomarkers may be identified based on their changes in protein modifications rather than changes in protein abundance.\textsuperscript{3,13,15}

\section*{CONCLUSIONS}

Our study shows an effective method for the analysis of glycosylation changes on glycoproteins from serum samples. This method can be used not only to analyze the high-abundance serum proteins, but also to quantify moderate-abundance proteins that are potential markers, as described in our previous study.\textsuperscript{15} Its effectiveness for low abundance glycoproteins has yet to be determined. Nevertheless, using this novel reverse lectin-based ELISA assay, we found that fucosylated haptoglobin could be a potential candidate biomarker, which can be used to supplement CA125 for detecting ovarian cancer with improved sensitivity and specificity. The method has distinct advantages over current lectin-ELISAs that require oxidation of glycans on the IgGs to minimize interaction with the lectins. However, even with this procedure, there is often significant background in these lectin-ELISA experiments compared with our current platform.

In future work, there are various ways in which the reverse lectin-based ELISA method could be expanded and improved. It could be expanded to a 384 well format to increase the throughput for a larger number of samples. Also, there has been recent work to improve the response of lectin arrays based on oriented lectins using an immobilization method.\textsuperscript{56–52} These oriented lectin arrays have been shown to markedly increase the response of some lectins to glycoproteins. AAL and SNA though were not tested in this work. Nevertheless, this strategy has worked for many lectins and could help in expanding this method to lower abundance glycoproteins.

\section*{ASSOCIATED CONTENT}

\section*{Supporting Information}

Determining the presence of fucosylation/sialylation of haptoglobin and IgG by Lectin-blot. Determining the specificity of the reverse lectin-based ELISA assay by detecting IgG. Evaluating the specificity of anti-haptoglobin and anti-IgG for the reverse lectin-based ELISA assay with depleted/original serum samples incubation. Levels of sialylated haptoglobin, sialylated IgG, and fucosylated IgG in sera of patients with ovarian cancer determined by reverse lectin-based ELISA assays. Detection of glycosylation on proteins after omitting various components from the mixture of reverse lectin-based ELISA assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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\subsection*{Notes}

The authors declare no competing financial interest.

\section*{ACKNOWLEDGMENTS}

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\section*{ABBREVIATIONS:}

AAL, aellura aurantia lectin; SNA, Sambucus nigra lectin; ELISA, enzyme-linked immunosorbent assay; CA125, cancer antigen 125; HRP, horseradish peroxidase; TMB, 3,3',5,5'-tetramethylbenzidine; ROC curve, receiver operating characteristic curve; AUC, area under an ROC curve

\section*{REFERENCES}

\begin{enumerate}
\item (15) Wu, J.; Xie, X.;Nie, S.; Buckanovich, R. J.; Lubman, D. M. Altered expression of sialylated glycoproteins in ovarian cancer sera
\end{enumerate}


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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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

![Figure 1. Workflow for candidate biomarker identification and validation.](dx.doi.org/10.1021/pr300330z)
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 intricately 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 IgG-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 underwent buffer exchange using an YM-3 centrifugal device (Millipore Corp., Bedford, 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 Ultragel 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 1-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-
man.v3.49). 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...
and the best-

cance of changed proteins between ovarian cancer and benign diseases after Student’s t-test.

from BioVendor (Candler, NC, USA). ELISA kits for SAP, CFAB, and HRG were from USCNK (Wuhan, China). CA125 ELISA kit was purchased from Genway (San Diego, CA, USA). ELISA assays were performed following the manufacturer’s instructions. The absorbance values were read on a microplate reader (BioTek, Synergy HT) at a wavelength of 450 nm.

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 between cancer and benign where the data were of borderline significance (p = 0.06) as shown in Figure 2b. Three of the lectins (LCA, UEAI, and AAL) have carbohydrate

Table 2. Significantly Changed Proteins between Stage III Ovarian Cancer and Benign Diseases Detected by Exactag Labeling Based Quantitative LC−MS/MS Analysis

<table>
<thead>
<tr>
<th>protein ID</th>
<th>protein name</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</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 prostestins 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 complement system</td>
</tr>
<tr>
<td>Q66PD5</td>
<td>N-acetylmuramoyl-L-Alanine 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>

<sup>a</sup>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 Confinatory Sample Set I

<table>
<thead>
<tr>
<th>protein</th>
<th>fucosylated protein levels&lt;sup&gt;b&lt;/sup&gt;</th>
<th>underlying protein concentrations (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean (range) mean (range)</td>
<td>mean (range) mean (range)</td>
</tr>
<tr>
<td></td>
<td>stage III stage I/II benign normal</td>
<td>stage III stage I/II benign normal</td>
</tr>
</tbody>
</table>

<sup>b</sup>Fucosylation levels were quantitated by absorbance at 450 nm (OD values) with a microplate reader. <sup>a</sup>p < 0.05 for differentiating between stage III and benign/normal (fucosylation); for difference between stage III and benign/normal and stage I/II and normal (underlying protein). <sup>b</sup>p < 0.05 for differentiating between stage III and benign/normal (fucosylation); for difference between stage III and benign/normal (underlying protein). <sup>c</sup>p < 0.05 for differentiating between stage III and benign/normal (fucosylation); for difference between stage III and stage I/II and normal (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 Neu5Acα2-6Gal (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. (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.](image-url)
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.

Figure 5. Verification of fucosylated protein changes using an independent sample set from GOG. (a) 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; (b) ROC curve for fucosylated CFAB to distinguish early stage cancer from benign diseases or healthy controls.
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). CA12S 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 (Figure 7a). The AUC for CBG, HRG, and SAP were 0.887, 0.811, and 0.687, respectively. CA12S obtained the highest AUC (0.936) for differentiation of stage III cancer from healthy controls. The combination of CBG, SAP, and HRG had an AUC of 0.949 with specificity of 100% at a sensitivity of 83%, which outperforms CA12S to distinguish stage III ovarian cancers from healthy controls.

CBG and SAP levels were found to be significantly changed between stage III cancer and benign diseases. The concentrations of CBG decreased from a mean of 19.25 µg/mL (range, 12.63–28.79 µg/mL) in benign diseases to 17.06 µg/mL (range, 11.15–24.72 µg/mL) in stage III ovarian cancer ($p < 0.05$), while SAP concentrations increased from a mean of 199.2 µg/mL (range, 36.01–368.0 µg/mL) in benign diseases to 259.2 µg/mL (range, 67.86–399.6 µg/mL) in stage III ovarian cancer ($p < 0.05$) (Figure 6). The ROC curves for CBG and SAP were constructed to distinguish stage III ovarian cancer from benign diseases. The AUC for CBG and SAP was 0.664 and 0.655, respectively. The clinically used biomarker CA12S obtained the highest AUC (0.821) for distinguishing stage III cancer from benign disease (Figure 7b). However, the combination of CBG, SAP, and CA12S resulted in an AUC of 0.846 with specificity of 63% at a sensitivity of 83%, which improved both sensitivity and specificity when compared to CA12S alone.

Importantly, protein levels of CBG and HRG significantly decreased in stage I/II cancer sera compared to healthy controls. ROC curves for CBG and HRG to distinguish stage I/II ovarian cancer from healthy controls are shown in Figure 7c. The AUC values for CBG and HRG were 0.667 and 0.819, respectively. The combination of CBG and HRG had an AUC of 0.825, which was comparable to CA12S (AUC = 0.829) to distinguish stage I/II ovarian cancer from healthy controls. With this initial sample set, we did not find any protein, which could be effectively used to distinguish stage I/II ovarian cancer from benign diseases.

Ultimately, 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 CA12S 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, in this study, 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
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.\textsuperscript{39,40} However, the expression of SAP in plasma of ovarian cancer patients has not been studied. Furthermore, in our previous study,\textsuperscript{41} 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.\textsuperscript{42−44} 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\textsuperscript{45} and pancreatic cancer.\textsuperscript{46} However, the roles of CFAB in the progress of cancer are not well understood. In the present study, we identified and confirmed that fucosylated glycosylation changes of CBG and SAP by using the independent sample set from GOG. (a) Protein levels were determined by ELISA assay. Protein levels with significant changes between pairwise comparisons are shown ($p < 0.05$). (b) ROC analyses for CBG, SAP, and CA125 to differentiate ovarian cancer from healthy controls or benign diseases.

Figure 8.

![Figure 8](image-url)

 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.

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![Figure 8](image-url)
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-acetylmuramyl-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

**REFERENCES**


