Proteomic-Based Discovery and Characterization of Glycosylated Eosinophil-Derived Neurotoxin and COOH-Terminal Osteopontin Fragments for Ovarian Cancer in Urine

Bin Ye,1 Steven Skates,3 Samuel C. Mok,5 Nora K. Horick,3 Helene F. Rosenberg,5 Allison Vitonis,1 Dale Edwards,1 Patrick Sluss,4 Won K. Han,2 Ross S. Berkowitz,1 and Daniel W. Cramer1

Abstract

Purpose: The objective was to identify and characterize low molecular weight proteins/peptides in urine and their posttranslational modifications that might be used as a screening tool for ovarian cancer.

Experimental Design: Urine samples collected preoperatively from postmenopausal women with ovarian cancer and benign conditions and from nonsurgical controls were analyzed by surface-enhanced laser desorption/ionization mass spectrometry and two-dimensional gel electrophoresis. Selected proteins from mass profiles were purified by chromatography and followed by liquid chromatography-tandem mass spectrometry sequence analysis. Specific antibodies were generated for further characterization, including immunoprecipitation and glycosylation. Quantitative and semiquantitative ELISAs were developed for preliminary validation in patients of 128 ovarian cancer, 52 benign conditions, 44 other cancers, and 188 healthy controls.

Results: A protein (m/z = 17,400) with higher peak intensities in cancer patients than in benign conditions and controls was identified and subsequently defined as eosinophil-derived neurotoxin (EDN). A glycosylated form of EDN was specifically elevated in ovarian cancer patients. A cluster of COOH-terminal osteopontin was identified from two-dimensional gels of urine from cancer patients. Modified forms EDN and osteopontin fragments were evaluated in early-stage ovarian cancers and a combination of both resulted to 93% specificity and 72% sensitivity.

Conclusions: Specific elevated posttranslationally modified urinary EDN and osteopontin COOH-terminal fragments in ovarian cancer might lead to potential noninvasive screening tests for early diagnosis. Urine with less complexity than serum and relatively high thermodynamic stability of peptides or metabolites is a promising study medium for discovery of the novel biomarkers which may present in many nonurinary tract neoplastic diseases.

Among the gynecologic cancers in the United States, ovarian cancer is associated with the highest death rate largely because of its tendency to present at an advanced stage associated with poorer survival. The possibility that early detection could improve survival has been suggested by a pilot study using initial screening with serum CA 125 with follow-up ultrasound for those with elevated levels but was accompanied by a relatively low positive predictive value (1). Predictive value could be improved by a highly sensitive and specific test for ovarian cancer, and the initial report that the use of mass spectrometry to identify proteomic patterns could yield such a test was greeted with initial excitement (2). Since then, problems have become apparent with the clinical application of mass spectrometry to serum, including stability of the patterns affected by details related to processing and storage of the specimens, interference from more abundant serum proteins such as albumin, and range of detection confined to low molecular weight proteins. These potential weaknesses for the application of mass spectrometry to serum may be potential strengths for its application to urine. Urinary proteins/peptides tend to be relatively small in size with a higher thermodynamic stability (3) and less complex and interactive compared with the proteins present in serum (4, 5). In this article, we describe the application of mass spectrometry as well as two-dimensional gel electrophoresis toward the identification, characterization, and preliminary evaluation of two potential urine biomarkers for ovarian cancer.
Materials and Methods

Urine specimens. All specimens were collected and archived under protocols approved by the Human Subjects Committees of the Partners HealthCare System (Boston, MA). Urine samples were collected preoperatively from the women undergoing surgery for a “pelvic mass” at Brigham and Women’s Hospital and Massachusetts General Hospital. Urine specimens were available from 258 women before surgery, 128 of them proved to have epithelial ovarian cancer, 24 had benign ovarian cyst or tumors, 28 with endometriosis, 24 with other gynecologic cancers, 22 with inflammation (interstitial cystitis), 12 with renal cancer, and 20 with other nongynecologic cancer. A total of 188 age-matched urine specimens collected from healthy women, including 100 randomly collected from our ongoing case-control study of ovarian cancer in Massachusetts and New Hampshire (6) and 88 from general population, were used as control. All specimens were collected in sterile containers, processed within 8 hours, and stored in 0.5%, 1.5%, and 15-mL aliquots at −80°C.

Mass spectrometry protein/peptide profiling. Thawed urine samples were thoroughly mixed followed by a centrifugation at 12,000 rpm at 4°C for 5 minutes. Based on the acidic nature of urine, the WCX2 (weak cation exchange, Ciphergen Biosystem, Fremont, CA) protein chip was selected for application with surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). The chips were first activated by 10 mmol/L of HCl for 5 minutes and then washed twice with 200 μL binding buffer (100 mmol/L ammonium acetate, pH 6.5) on the bioprocessor. Fifty microliters of the urine sample were directly applied on the spot surface and the chip was inserted into a bioprocessor for incubation on the mini-shaker (IKA-Work, Inc., Wilmington, NC) at 600 rpm at room temperature for 1 hour. After two washes with 350 μL of the binding buffer, the air-dried arrays were then treated with saturated 3.5% dimethyloctahydroxyinnamic acid (sinapinic acid) in 0.5% trifluoroacetic acid and 50% acetonitrile before being analyzed by SELDI-TOF-MS (Protein Biology System II, Ciphergen Biosystems). The detailed setting of mass resolution, accuracy, calibration, and shooting protocol followed our previously reported protocol (7).

Two-dimensional gel electrophoresis of urine proteins/polypeptides. Pooled urinary protein samples, grouped by case and control status, were desalted by Sephadex G-25 column. Lipids and nucleic acids were removed using a commercial protein clean up kit (Bio-Rad, Hercules, CA). Briefly, the concentrated urine protein (~500 μg) was added with 300 μL of the provided precipitating agent 1, mixed well, followed by incubation for 15 minutes in ice. After adding 300 μL of precipitating agent 2 and mix by vortex, samples were spin for 5 minutes at >12,000 × g to form the protein pellet. By two washes with washing buffers I and II, the protein pellets were dissolved in a 15-mL aliquots at −80°C. After gel staining, the protein/polypeptide identity was searched from the SWISS-PROT database.

Protein purification. Three milliliters of urine from an ovarian cancer patient, with high intensity peaks at 8,700 and 17,400 Da on SELDI-MS, were filtered through a 0.45-μm membrane. An equal volume of PBS buffer containing 1.0% CHAPS and 8 mol/L urea was mixed with urine sample. After desalting with Sephadex G-25 column, the filtered sample was loaded into an ion exchange column (1.0 mL, HiTrap CM-FF, Amersham Bioscience Corp., Piscataway, NJ) for purification. Elution buffers of 100 mmol/L ammonium acetate (pH 6.0) with gradient concentration of sodium chloride (0-1.0 mol/L) were used. The eluted fractions of interest were first subjected to SELDI-MS to confirm the protein peak and were separated by 10% to 20% gradient SDS-PAGE. The separated proteins were visualized by silver staining.

Protein identification. After gel staining, the protein/polypeptide bands or spots of interest were excised from gels for sequence analysis using liquid chromatography with online sequence analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS; refs. 8, 9), with LCD DECAXP plus ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA) at Taplin Biological Mass Spectrometry Facility, Harvard Medical School. The fragmentation ladders (the b- and y-ion series) from the lowest mass to the highest mass were used for the identification of the amino acid residues of the peptides and the protein identity was searched from the SWISS-PROT database.

Antibody generation and Western blotting. Based on the predicted antigenicity from the amino acid sequence, the COOH-terminal peptide RDPQQYTVVPHDLR generated from the LC/MS/MS analysis of the protein correlating with the SELDI peak was submitted for peptide synthesis. The synthesized peptide was conjugated with keyhole limpet hemocyanin and applied as antigen to generate rabbit polyclonal antibodies (Invitrogen Corp., Carlsbad, CA). The affinity-purified antibody was then used for antigen-antibody titration analysis and ELISA assay.

Western blotting: Samples with 6 μg urinary protein were prepared by acetone precipitation from control, benign, and cancer patients and subjected to 8% to 16% SDS-PAGE separation. Purified human eosinophil-derived neurotoxin (EDN) overexpressed in E. coli was used as positive control (10). After proteins were transferred to a polyvinylidene difluoride membrane, 5% (w/v) of fat-free milk in TBST [10 mmol/L Tris-HCl/100 mmol/L NaCl/0.1% (v/v) Tween 20 (pH 7.5)] was used for blocking overnight at 4°C. The polyclonal antibody against human EDN was used for the primary reaction at a 1:200 dilution in TBST with 5% (w/v) fat-free milk for 2 hours. The membrane was then washed thrice with TBST-Tween 20 (TBST) for 15 minutes per wash. The secondary antibody was an anti-rabbit immunoglobulin G coupled to horseradish peroxidase and was used according to a protocol for enhanced chemiluminescent detection (Pierce Biotechnology, Inc., Rockford, IL).

A total of 25 μg urinary protein prepared from patients with serous invasive ovarian cancer were used for two-dimensional gel-based Western blotting. Urine protein aliquots (about 5 μg each) prepared from stored specimens of normal women, benign, and cancer patients were subjected to 8% to 16% SDS gel separation for the regular one-dimensional gel Western blot. The proteins/polypeptides on the gels were subjected to 8% to 16% SDS gel separation for the regular one-dimensional gel Western blot. The proteins/polypeptides on the gels were then transferred to polyvinylidene difluoride membrane and blocked with 5% (w/v) fat-free milk in TBST. A monoclonal antibody against human osteopontin (Immunno-Biological Lab Co., Takasaki-Shi, Gunma, Japan) was used as primary antibody at 1:3,000 in TBST with 5% fat-free milk for 2 hours. The secondary antibody application and visualization of osteopontin expression in urine specimens of cancer and normal controls followed the method as described above.

Immunoprecipitation. A total of 400 μg urine protein prepared from acetone precipitation were desalted by Sephadex G-25 column and incubated with 10 μL polyclonal antihuman EDN antibody in the buffer [20 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1 mmol/L MgCl2, 0.5% NP40 and 10% glycerol] for 2 hours at 4°C. Twenty microliters of Protein G (Pierce) were added for the antigen-antibody binding at 4°C for overnight. The incubation mixture was centrifuged at 14,000 rpm, 4°C for 5 minutes. The precipitated pellets were washed...
thrice with the above-mentioned buffer by centrifugation. The preimmune rabbit serum was applied as a negative control for nonspecific binding. The immunoprecipitated protein pellets were dissolved in 20 μL of protein sample buffer which contained 187 mmol/L Tris-HCl (pH 6.8), 30% glycerol, 15% β-mercaptoethanol, and 9% SDS. After boiling for 5 minutes, the proteins were separated on the 15% SDS-PAGE.

Protein glycosylation analysis. N-Glycanase (peptide-N-glycosidase F) and O-glycanase (Prozyme, Inc., San Leandro, CA) were used to pretreat equal amounts (10 μg) of urine protein from patients with benign and malignant ovarian masses for 2 hours at 37°C water bath. The enzymes and reaction buffers were added according to the provided protocol. After glycanase pretreatment, proteins were separated by SDS-PAGE followed by glycoprotein staining with Pro-Q Emerald 300 (Molecular Probes, Eugene, OR). Identical gels were used for Western blot to identify the corresponding glycoprotein on the gels.

ELISA quantification. To detect total urine EDN including glycosylated forms, a polyclonal antibody generated against the synthetic peptide was used for ELISA assay. Briefly, urine specimens were diluted (1:10) in water containing 0.2% SDS, followed by heating at 100°C for 3 minutes. After the sample was cooled, the denatured urine specimens (duplicates for one specimen, and 100 μL for each) were coated on a 96-well microplate overnight at room temperature. Purified synthetic EDN peptide was applied as a standard antigen for concentration calibration. After washing thrice with buffer (5 mmol/L Tris-HCl, 0.15 mol/L NaCl, and 0.05% Tween 20), the testing sample and purified peptide standards were then incubated with the first antibody at dilutions of 1:100 and 1:1,000, respectively, in a buffer containing 50 mmol/L Tris-HCl (pH 7.5) with 0.1% SDS for 1 hour at 37°C. The antigen concentration was recovered using Turbo-TMB peroxidase was diluted (1:1,000) in 50 mmol/L Tris-HCl (pH 7.5) with 1 mol/L thiourea, 2% CHAPS, and 0.2% SDS. Sample was then loaded on the polyvinylidene difluoride membrane, which was inserted in a 96-well dot-blot cassette. Concentrations of 0, 0.05, 0.1, 0.2, 0.4, and 0.8 ng of mixture of human osteopontin and peptides were spotted on each blot for standard calibration. After blocking with 3% bovine serum albumin and antibodies against NH2-terminal of human osteopontin (Assay Designs, Inc., Ann Arbor, Michigan) for overnight at 4°C, the monoclonal antibody against COOH-terminal osteopontin (KHLFKRISHLDSASEVN; Assay Designs) at 1:500 dilution in 6% bovine serum albumin dilution buffer was used as first immune reaction at room temperature for 1 hour. After washing thrice, the second antibody conjugated with horseradish peroxidase was used to reveal the antigen concentration by chemiluminescent reaction. Less than 20% of SD (n = 20) was achieved in this assay. Each sample was run in duplicate and three individual cancer cases were used for internal calibration.

Urinary protein/peptide quantification and normalization. The total protein concentration in each urine specimen was measured by the bicinchoninic acid assay according to the protocol provided by Pierce and used to normalize the EDN and osteopontin concentration, which was defined as micrograms or nanograms per milligram of protein. The urine protein samples applied for Western blot and immunoprecipitation were first concentrated by acetone precipitation and quantified with a Bradford method with bovine serum albumin as standard.

![Fig. 1. A, urinary protein/peptide profiling by SELDI-mass spectrometry. Profiles of mass range of 1,000 and 20,000 were displayed according to mass-to-charge ratio (m/z). The peaks of interest at 8,730 (dashed arrow) and 17,400 m/z (solid arrow) were shown in ovarian benign tumor and cancer patients but less detectable in normal healthy women. The means and SEs of peak intensities of profiles in normal, benign, and cancer patients were summarized. B, the identical peak pattern of interest at 8,730 and 17,400 m/z was shown in a cancer case before (top) and after Hifrap CM-FF ion exchange column purification (bottom). C, SDS-PAGE separation and silver staining of the purified protein of interest. About 10 μg of c oncentrated original urine protein (lane 1) and the enriched purified protein fraction (~3 μg, lane 2) were separated on 15% SDS gel. The high-mobility band (arrow) of the protein of interest corresponding to 17,400 Da was indicated.](www.aacrjournals.org)
Data set to obtain variable estimates and then uses these estimates to validate. This technique iteratively omits one observation from the separate validation data set, we used the “leave-one-out” cross-validation to evaluate the sensitivity and specificity predicted by the models without using a changes in sensitivity within the highest specificity regions. To validate

false-positive rates (0-10% for the case versus control comparison and 0-50% for the case versus benign comparison) to clearly indicate

Graphs of receiver operator characteristic curves focused on acceptable cancer cases from healthy controls and ovarian cancer from benigns. Receiver operator characteristic curves were generated to evaluate the sensitivity of EDN and COOH-terminal osteopontin biomarkers, both individually and in concert, for distinguishing ovarian cancer cases from healthy controls and ovarian cancer from benigns. Graphs of receiver operator characteristic curves focused on acceptable false-positive rates (0-10% for the case versus control comparison and 0-50% for the case versus benign comparison) to clearly indicate changes in sensitivity within the highest specificity regions. To validate the sensitivity and specificity predicted by the models without using a separate validation data set, we used the “leave-one-out” cross-validation. This technique iteratively omits one observation from the data set to obtain variable estimates and then uses these estimates to classify the omitted subject as a case or control.

Results

Urinary protein/peptide SELDI-MS profiling and liquid chromatography purification. Figure 1A illustrates a commonly observed pattern with application of SELDI-TOF-MS to the urine spotted on WCX chip surfaces. Two protein peaks at 8,730 and 17,400 m/z were present in the urine of ovarian cancer cases but less frequently in controls. These two peaks were defined as one protein identity by using the software analysis based on mass-to-charge ratio calculation (8,730 and 17,410 are of double and single charges, respectively). Differential intensities of urine profiles corresponding to the two peaks in normal, benign, and cancer patients were summarized. The mean values of peak intensities in cancer patients were significantly higher than benign and controls (P < 0.001; Table 1). Figure 1B shows the results of protein purification using the cationic ion exchange chromatography. Most of the urinary proteins/small peptides at <50 kDa may also be present). Two-dimensional gel profiling of urinary proteins/peptides in ovarian cancer subtypes. Figure 2 presents the results of the two-dimensional electrophoresis applied to the pooled urine proteins from controls, those with benign disease, and those with different histologic types of cancer (serous invasive, mucinous, and endometrioid/clear cell). At the range of mass <40 kDa and pH 4 to 7.5, one cluster of protein spots (Fig. 2C-E) was consistently shown in ovarian cancer subtypes, but none or much less was found in benign and normal controls (Fig. 2A and B).

LC-MS/MS–based protein identification of EDN and osteopontin. The purified urinary protein fraction containing 8,700 and 17,400 m/z was separated by SDS gel and the corresponding protein band at 17,400 Da was excised from the observed molecular weight of ~17 kDa on the gel (a second form of higher molecular weight at position of ~35 kDa may also be present).

Table 1. Summary of peak intensities of urine protein profiles

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total no.</th>
<th>8,700 Da Mean (SE)</th>
<th>17,400 Da Mean (SE)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>55</td>
<td>0.41 (0.26)</td>
<td>0.55 (0.12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Benign</td>
<td>48</td>
<td>5.16 (0.93)</td>
<td>&lt;0.001</td>
<td>7.72 (0.94)</td>
</tr>
<tr>
<td>Cancer</td>
<td>42</td>
<td>10.90 (2.00)</td>
<td>&lt;0.001</td>
<td>13.80 (2.30)</td>
</tr>
</tbody>
</table>

Fig. 2. Urinary protein/peptide profiling by two-dimensional gel electrophoresis. A total of 50 µg urinary protein of age-matched groups (50 years old) of healthy women (A), benign conditions (B), ovarian cancer patients with different subtypes of serous invasive (C), mucinous (D), and endometrioid/clear cell (E) were separated by two-dimensional electrophoresis followed by SYPRO-Ruby staining. Protein spots at a range of 400 kDa and pH 4 to 7.5 were displayed. One cluster of protein/polypeptide spots partially indicated by a circle appeared in ovarian cancer patients only.
the gel and subjected to trypsin digestion. The resultant peptides were further subjected to LC-MS/MS for sequence analysis. The identity of this cationic protein was defined as EDN (also known as RNase 2) with the COOH-terminal peptide sequence RDPPQYPVVPVHLDR (Fig. 3A). Three protein spots of the indicated cluster with SYPRO-Ruby staining were excised from a two-dimensional gel and subjected to trypsin digestion followed by LC-MS/MS sequence analysis. Only one protein, osteopontin, was matched to the sequences of all five peptides (Fig. 3B; Table 2). It was defined as the COOH-terminal fragments of osteopontin.

**Glycosylation of urinary EDN.** EDN contains five potential asparagine residues for N-linked glycosylation (13, 14), suggesting that differential glycosylation could be the source of the molecular weight differences and two peaks of m/z in the mass profiles. Western blotting was used to detect urine EDN of the cancer patients with or without N-glycanase treatment. Figure 4A showed that the high molecular weight, lower-mobility hyperglycosylated form of EDN (see upper arrow II) was transformed to lower molecular weight, high-mobility form (see arrow I) after glycanase treatment. The lower molecular weight, high-mobility form of EDN was also slightly shifted to an even smaller size after treatment. This indicated that the native monomer EDN was likewise slightly or minimally glycosylated. Immunoprecipitated EDNs from pooled urine specimens of the benign tumor and cancer patients were detected by Western blot and revealed a relatively greater intensity of hyperglycosylated form of EDN in ovarian cancer patients (Fig. 4B, upper band II). The minimally glycosylated EDN (lower band I), however, appeared with similar intensities in the ovarian cancer patients and those with benign conditions (Fig. 4B, left). When the identical gel was submitted for glycosylation staining, the upper and presumably hyperglycosylated band of EDN indeed showed much greater intensity in ovarian cancer than in benign patients (Fig. 4B, right). There was much less detectable difference in glycosylation intensity of the lower molecular weight form of EDN. We have done immunoprecipitation to enrich urinary EDN from individual specimens of the benign tumor (n = 18) and ovarian cancer patients (n = 20) and confirmed that the hyperglycosylated form of EDN (upper band II) has a greater intensity (2-fold, P < 0.001) in cancer patients than in those with benign conditions (Fig. 4C and D).

**Osteopontin fragmentation.** To confirm the protein identification and differential expression of osteopontin, we have used two-dimensional gel electrophoresis–based Western blots to analyze the urine specimens obtained from normal, benign, and cancer patients. The identity of the cluster spot pattern was confirmed as COOH-terminal osteopontin peptides and was revealed on the two-dimensional gel Western blot (Fig. 4E). The pattern was not identified in the normal and benign urine specimens, which was consistent with the original protein

### Table 2. Identity of the COOH-terminal domains and peptides of urinary osteopontin in ovarian cancer patients

<table>
<thead>
<tr>
<th>Position</th>
<th>MH+</th>
<th>Sequence</th>
<th>Scores (XCorr/dCn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>302-314</td>
<td>1,388.4307</td>
<td>ISHELDASSEVYN</td>
<td>3.28/0.24</td>
</tr>
<tr>
<td>250-268</td>
<td>2,118.1321</td>
<td>ANDESNEHDVIDSQELSK</td>
<td>4.46/0.43</td>
</tr>
<tr>
<td>223-241</td>
<td>2,179.1750</td>
<td>DSYETSQLDGSAETHSHK</td>
<td>5.17/0.38</td>
</tr>
<tr>
<td>221-241</td>
<td>2,364.4009</td>
<td>GKDYSQTDGSAETHSH</td>
<td>5.84/0.48</td>
</tr>
<tr>
<td>176-203</td>
<td>3,226.4092</td>
<td>RPDIQYPDATEDITSHMEELNGAYK</td>
<td>5.99/0.44</td>
</tr>
</tbody>
</table>

NOTE: Five peptide sequences, positions, and matched scores were determined with LC-MS/MS analysis and located at the COOH-terminal domain of intact protein.
profiling (Fig. 2). Indeed, our one-dimensional gel Western blot also showed that at least two protein fragments at molecular weight of ~20 kDa were present only in ovarian cancer patients, but not in benign and normal controls (Fig. 4F). Similar osteopontin peptide patterns were also detected in sera of ovarian cancer patients (data not shown). Interestingly, we have previously identified osteopontin as a potential serum biomarker for ovarian cancer by using the DNA microarray technology (15). Here we confirm that osteopontin may be a valuable marker for ovarian cancer and extended our finding that osteopontin-derived COOH-terminal fragments may be more specific and sensitive for ovarian cancer detection.

ELISA quantification of urinary EDN and osteopontin. Although specific antibodies for hyperglycosylated EDN and osteopontin COOH-terminal fragments are not yet available, we have used the home-made polyclonal antibody against EDN peptide, commercial osteopontin assay kit, and a monoclonal antibody against one synthetic COOH-terminal peptide (KHLKFRISHELDSASEVN) for a preliminary evaluation using 412 urine specimens. The average (SD) of age for ovarian cancer cases, other cancers, benign conditions, urine tract inflammation, and normal group were 55.8 (11.8), 60.3 (13.5), 52.2 (14.4), 62.6 (15.4), and 53.5 (11.5), respectively. After normalization by total urinary protein, the geometric mean values of EDN level (ng/mg protein) were 4.81 for 128 cancer patients, 1.97 for 44 other cancers, 1.36 for 52 benign conditions, 0.28 for 22 bladder inflammatory conditions (interstitial cystitis), and 0.93 for 188 normal healthy controls (Table 3; Fig. 5A). Mean EDN level in the ovarian cancer group was significantly higher than that in normal controls (5.2-fold, \( P < 0.001 \)), urinary tract inflammation (17.1-fold, \( P < 0.001 \)), benign conditions (3.5-fold, \( P < 0.001 \)), and other cancers (2.4-fold, \( P < 0.001 \)). The mean value of urinary EDN in stage I ovarian cancer patients was the highest (5.95) compared with other stages (4.06 for stage II and 4.47 for stage III/IV). Among histologic subtypes of ovarian cancer, serous borderline cancer patients exhibited the highest mean EDN level of 6.43 (Table 3).

**Fig. 4.** A, glycanase pretreatment and Western blot detection of the minimally (I) and hyperglycosylated (II) EDN forms in urine of ovarian cancer patients. B, Western blot analysis and glycosylation staining of the immunoprecipitated urine EDN forms (I and II) in benign and cancer patients. C, hyperglycosylated EDN (II) in benign and cancer patients detected by Western blot. Purified recombinant human EDN expressed from E. coli (2 μg) was used as positive control (left lane). D, summary of hyperglycosylated (II) EDN elevated in cancer patients \( (n = 20) \) compared with benign patients \( (n = 18) \). *, \( P < 0.001 \) (ANOVA). E, elevated cluster of osteopontin fragments was shown by SYPRO-Ruby staining and two-dimensional gel Western blot analysis. F, differential expression of urinary osteopontin peptides (~20 kDa) in normal, benign, and ovarian cancer patients.
Osteopontin was measured in the same set of urine specimens and yielded geometric mean values of 0.78, 2.29, 0.94, and 2.90 µg/mg protein in normal healthy controls, benign ovarian conditions, other cancers, and ovarian cancers, respectively. The mean value of osteopontin in ovarian cancers was significantly higher than that in other cancers (∗P = 0.03) and normal controls (∗∗P < 0.001). Interestingly, mucinous (mean = 4.42) and serous borderline (mean = 4.02) subtypes exhibited the highest urinary osteopontin levels compared with other ovarian cancer subtypes. The COOH-terminal osteopontin levels (ng/mg protein) showed much better separation of ovarian cancer group (2.92) from inflammatory conditions (0.07), benign conditions (0.94), other cancer group (0.25), and normal control (0.41; Table 3; Fig. 5C). This was consistent with previous Western blot data (Fig. 4F) and confirmed that COOH-terminal osteopontin fragments may be more specifically elevated in ovarian cancer patients. In addition, elevated COOH-terminal osteopontin in urine was strongly correlated with ovarian cancer metastasis, with mean levels of 1.94, 2.91, and 3.64 in stage I, stage II, and late-stage (III-IV) patients, respectively (Table 3).

**Combination of urinary EDN and COOH-terminal fragment of osteopontin.** Figure 5D displayed a receiver operator characteristic curve for EDN, COOH-terminal osteopontin, and the two markers together, and illustrated that using both biomarkers together to predict ovarian cancer status results in substantially increased sensitivity at any given level of false-positive rate across the 0% to 10% percent range. Specifically, combining both biomarkers yields 72% sensitivity at the 95% specificity level (i.e., 5% false-positive rate) to distinguish ovarian cancer from normal controls, compared with 47% for COOH-terminal osteopontin fragments alone and 63% for EDN alone at the same specificity. The combined ability of two markers to distinguish early-stage ovarian cancer cases from healthy controls was also evaluated. Figure 5F presents a bivariate scatterplot of the COOH-terminal osteopontin and EDN with a decision line and resulted to 72% sensitivity at 93% specificity. At a fixed specificity level

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients</th>
<th>EDN (ng/mg protein)</th>
<th>Osteopontin (µg/mg protein)</th>
<th>C-Osteopontin (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Geometric mean (SE)</td>
<td>P*</td>
<td>Geometric mean (SE)</td>
</tr>
<tr>
<td>Normal</td>
<td>188</td>
<td>0.93 (0.18)</td>
<td>&lt;0.001</td>
<td>0.78 (0.29)*</td>
</tr>
<tr>
<td>Population controls</td>
<td>100</td>
<td>0.78 (0.24)</td>
<td>0.70 (0.58)</td>
<td>0.27 (0.48)</td>
</tr>
<tr>
<td>MGH controls</td>
<td>88</td>
<td>1.12 (0.26)</td>
<td>0.89 (0.21)</td>
<td>0.65 (0.24)</td>
</tr>
<tr>
<td>Inflammation disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interstitial cystitis</td>
<td>22</td>
<td>0.28 (0.99)</td>
<td>&lt;0.001</td>
<td>0.07 (3.16)</td>
</tr>
<tr>
<td>Benign</td>
<td>52</td>
<td>1.36 (0.48)</td>
<td>&lt;0.001</td>
<td>2.29 (0.44)</td>
</tr>
<tr>
<td>Endometrioma</td>
<td>28</td>
<td>1.02 (0.81)</td>
<td>2.98 (0.74)</td>
<td>1.33 (0.68)</td>
</tr>
<tr>
<td>Ovarian cyst</td>
<td>8</td>
<td>1.82 (0.67)</td>
<td>1.80 (0.66)</td>
<td>0.19 (1.06)</td>
</tr>
<tr>
<td>Ovarian tumor</td>
<td>12</td>
<td>1.68 (0.77)</td>
<td>1.50 (0.78)</td>
<td>1.35 (1.74)</td>
</tr>
<tr>
<td>Other†</td>
<td>4</td>
<td>2.95 (0.70)</td>
<td>2.10 (0.79)</td>
<td>0.71 (1.16)</td>
</tr>
<tr>
<td>Other cancer</td>
<td>56</td>
<td>1.97 (0.63)</td>
<td>&lt;0.001</td>
<td>0.94 (1.42)†</td>
</tr>
<tr>
<td>Other non-gynecologic cancer</td>
<td>20</td>
<td>2.18 (0.87)</td>
<td></td>
<td>0.25 (1.10)</td>
</tr>
<tr>
<td>Renal cancer†</td>
<td>12</td>
<td>1.42 (1.72)</td>
<td></td>
<td>0.49 (3.14)</td>
</tr>
<tr>
<td>Other gynecologic cancer</td>
<td>24</td>
<td>1.80 (0.92)</td>
<td>0.94 (1.42)</td>
<td>0.19 (1.74)</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>128</td>
<td>4.81 (0.23)</td>
<td>2.90 (0.35)</td>
<td>2.92 (0.38)</td>
</tr>
<tr>
<td>Serous borderline</td>
<td>15</td>
<td>6.43 (0.88)</td>
<td>4.02 (0.78)</td>
<td>2.21 (1.28)</td>
</tr>
<tr>
<td>Serous invasive</td>
<td>69</td>
<td>4.43 (0.31)</td>
<td>2.64 (0.46)</td>
<td>2.97 (0.49)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>19</td>
<td>4.78 (0.50)</td>
<td>4.42 (0.74)</td>
<td>2.22 (1.56)</td>
</tr>
<tr>
<td>Endometrioid/clear cell</td>
<td>16</td>
<td>5.12 (0.67)</td>
<td>2.06 (1.71)</td>
<td>3.80 (0.83)</td>
</tr>
<tr>
<td>Other/undifferentiated†</td>
<td>9</td>
<td>4.99 (0.54)</td>
<td>2.57 (1.71)</td>
<td>4.53 (1.08)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>39</td>
<td>5.95 (0.43)</td>
<td>3.81 (0.48)</td>
<td>1.94 (0.75)</td>
</tr>
<tr>
<td>II</td>
<td>16</td>
<td>4.06 (0.57)</td>
<td>2.34 (1.40)</td>
<td>2.91 (1.39)</td>
</tr>
<tr>
<td>III-IV</td>
<td>73</td>
<td>4.47 (0.30)</td>
<td>2.64 (0.50)</td>
<td>3.64 (0.45)</td>
</tr>
</tbody>
</table>

Abbreviation: MGH, Massachusetts General Hospital.

†Student’s t tests (with correction for nonequal variances) were used to compare (log-transformed) mean values in normal, benign, and other cancer groups to mean levels in ovarian cancers (reference group).

††Other benign conditions are two for inflammatory uterus or salpingitis and two for torsion of ovary or tube.

‡‡There were only 14 urine specimens of other gynecologic cancer available when we purchased ELISA kit.

†††Preoperative female patients of renal clear cell carcinoma with elective nephrectomy.

| Other cancer types/undifferentiated patients include five undifferentiated, three transitional cell types, and one with the mixed serous and transitional types.
of 95%, 65% sensitivity was achieved under “leave-one-out” cross validation.

**Discussion**

The application of two distinct methods for proteomic analysis of urine obtained from women undergoing surgery for a pelvic mass as well as control women from the general population has yielded two initial candidate biomarkers for ovarian cancer. By using SELDI-TOF-MS and two-dimensional gel electrophoresis, we identified modified EDN and a cluster of COOH-terminal osteopontin fragments as potential urine markers, respectively. Similar to the previously identified many serum cancer biomarkers with high abundance (16), none of these are at low abundant level. However, the COOH-terminal osteopontin fragments present in urine at the range of nanograms per milligram of protein, and far less than the intact osteopontin at the range of micrograms per milligram protein.

![Boxplots illustrating elevation of urinary EDN, osteopontin, and COOH-terminal osteopontin in ovarian cancer compared with normal controls, urine track inflammation, benign ovarian conditions, and other cancers.](image)

![Receiver operating characteristic curves showing the ability of EDN and COOH-terminal osteopontin to distinguish ovarian cancer cases from healthy controls and benign ovarian conditions. Only the portions of the curves corresponding to feasible false-positive rates for an early detection program are displayed. A baseline displaying no information in a test, where sensitivity equals false-positive rate, was added to avoid possible misinterpretation of the curves due to the restricted range of false-positive rates. Early-stage cases are separated from controls by a logistic regression model of EDN and COOH-terminal osteopontin. The equation for the line was 2.19 \times \log(EDN) + 0.50 \times \log(c\text{-terminal osteopontin}) = 2.62, which was obtained by substitution of a cutoff probability for predicted case/control status corresponding to 72% sensitivity and 93% specificity into the estimated regression equation.](image)
of protein. Our data also confirmed that the posttranslational modification, such as glycosylation (17, 18), and protease cleaved peptides (19, 20) are essentially associated with cancer progression.

EDN is a major secretory product of human eosinophilic leukocytes and is a pyrimidine-specific nuclease of RNase A gene superfamily, also known as RNase 2. The elevated urinary EDN levels in ovarian cancer patients likely derived from high serum levels but whether this represents eosinophilia or an aberrant tumor product is unknown. The former possibility seems more likely because eosinophilic counts were increased in ovarian cancer patients and significantly correlated with the high level of serum EDN and CA125 (data not shown). Eosinophilia was also found in many other types of cancers, including breast, lung and colorectal cancers (21–25). Samoszuk's group has shown the presence and degragation of eosinophilins in ovarian cancer tissues (26) and in endometriosis (27). An increased total RNase activity has been reported in women with ovarian carcinoma (28) and may be due to elevated EDN in serum because EDN has 100- to 1,000-fold more RNase activity than most other members of the RNase superfamily (29).

EDN has been observed to have an inhibitory effect on oocyte maturation in pregnant women (30) and in rats (31). Some of the EDN functions which specifically target to ovary may be carried out by conjugation with other hormones such as gonadotropins (32, 33) and other roles may be modified by the degree of glycosylation (14, 34). These findings may be relevant to our observation that EDN present in ovarian cancer patients seemed to be more heavily glycosylated.

Osteopontin is an arginine-glycine-aspartic acid glycoprotein and possesses multifunctional activities including regulation of immune cell and control tumor cell phenotype (35, 36). Interestingly, osteopontin is known to be a substrate for proteolytic cleavage by the proteases thrombin (37) and matrix metalloproteinases (38, 39). The cleaved lower molecular weight osteopontin fragments can penetrate into cells and have biological activities in promoting both cell adhesion and migration, but these are not observed for the intact osteopontin molecule (38). Thus, it was proposed that cleaved osteopontin may be a member of the SIBLING family of small integrin-binding ligand N-linked glycoproteins that can increase invasiveness of cancer cells by interacting with their specific matrix metalloproteinase and integrin partners, which was shown by a recent in vitro study (40). Our semiquantitative data clearly showed that COOH-terminal osteopontin fragments were strongly correlated with ovarian cancer stages or invasiveness and support the previous findings. It may further suggest that cleaved COOH-terminal osteopontin fragments could be a more sensitive biomarker with high specificity compared with the intact osteopontin, which may be associated with other benign and inflammatory diseases (41–45). Without the refined assays of these biomarkers, our preliminary assay has achieved 93% specificity and 72% sensitivity for early-stage ovarian cancers.

We suggested that both EDN and osteopontin, like many other identified abundant protein biomarkers (46) in ovarian cancer, such as apolipoprotein a-1 (47, 48), transthyretin fragment (48), and haptoglobin-α fragment (7), may be strongly associated with inflammation. However, our preliminary data showed that the immune reaction patterns of antibodies for C-osteopontin and glyco-EDN in urine specimens are very different from the patterns displayed in serum/or plasma (data not shown). We suggest that a small proportion of the “pooled multiform biomarkers” (i.e., osteopontin), which specifically modified and associated with cancer invasion, if presented in urine, will be of great value in the separation of cancer patients from the non-cancerous conditions, simply because of much less complexity and less immune cross-reactions in urine tests compared with blood tests.

Our study certainly revealed the great potential of using proteomic techniques to study urinary proteins and peptides for early detection of cancer. The high thermodynamic stability of lower molecular weight urinary proteins/peptides, which are less complex and have less molecular interaction compared with serum, makes them more suitable for study with mass spectrometric techniques for biomarker discovery. Small sizes of urine proteins/peptides with their posttranslational modifications and derived abnormal metabolites associated with many diseases, including nonurine tract cancer diseases, might be a great promise for noninvasive diagnosis and disease monitoring in the future.

References
Proteomic-Based Discovery and Characterization of Glycosylated Eosinophil-Derived Neurotoxin and COOH-Terminal Osteopontin Fragments for Ovarian Cancer in Urine

Bin Ye, Steven Skates, Samuel C. Mok, et al.


Updated version Access the most recent version of this article at: [http://clincancerres.aacrjournals.org/content/12/2/432](http://clincancerres.aacrjournals.org/content/12/2/432)

Cited articles This article cites 48 articles, 16 of which you can access for free at: [http://clincancerres.aacrjournals.org/content/12/2/432.full.html#ref-list-1](http://clincancerres.aacrjournals.org/content/12/2/432.full.html#ref-list-1)

Citing articles This article has been cited by 16 HighWire-hosted articles. Access the articles at: [http://clincancerres.aacrjournals.org/content/12/2/432.full.html#related-urls](http://clincancerres.aacrjournals.org/content/12/2/432.full.html#related-urls)

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.