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Proteomic analysis of stage I endometrial cancer tissue: Identification of proteins associated with oxidative processes and inflammation

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Abstract

Objective—The present study aimed to identify differentially expressed proteins employing a high resolution mass spectrometry (MS)-based proteomic analysis of endometrial cancer cells harvested using laser microdissection.

Methods—A differential MS-based proteomic analysis was conducted from discrete epithelial cell populations gathered by laser microdissection from 91 pathologically reviewed stage I endometrial cancer tissue samples (79 endometrioid and 12 serous) and 10 samples of normal

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Conflict of interest statement

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endometrium from postmenopausal women. Hierarchical cluster analysis of protein abundance levels derived from a spectral count analysis revealed a number of proteins whose expression levels were common as well as unique to both histologic types. An independent set of endometrial cancer specimens from 394 patients were used to externally validate the differential expression of select proteins.

Results—209 differentially expressed proteins were identified in a comparison of stage I endometrial cancers and normal post-menopausal endometrium controls ($Q < 0.005$). A number of differentially abundant proteins in stage I endometrial cancer were identified and independently validated by western blot and tissue microarray analyses. Multiple proteins identified with elevated abundance in stage I endometrial cancer are functionally associated with inflammation (annexins) and oxidative processes (peroxiredoxins). PRDX1 and ANXA2 were both confirmed as being overexpressed in stage I cancer compared to normal endometrium by independent TMA ($Q = 0.008$ and $Q = 0.00002$ respectively).

Conclusions—These data provide the basis for further investigation of previously unrecognized novel pathways involved in early stage endometrial carcinogenesis and provide possible targets for prevention strategies that are inclusive of both endometrioid and serous histologic subtypes.

Keywords

Endometrial cancer; Laser capture microdissection; Proteomics; Stage I

Background

According to the American Cancer Society, an estimated 43,470 new cases of uterine cancer were expected to be diagnosed in 2010 in the U.S., making this the fourth most common cancer in women and the most frequent type of gynecologic malignancy [1]. Recent data show a significant increase in the proportion of patients presenting with advanced stage endometrial cancers, particularly those with histologic subtypes associated with poor prognosis [2]. Despite continued improvements in contemporary therapeutics for other non-uterine gynecologic malignancies, there have been few improvements in the treatment of advanced stage endometrial cancer. While the 5-year survival for stage I disease is 83% only 17–20% of patients with distant metastasis will be cured of their disease. [1,3]. These data indicate that research is desperately needed to provide improved understanding of the molecular alterations associated with endometrial cancer in an effort to identify targets for future prevention and therapeutic treatment strategies.

Microarray studies have shown unique gene expression profiles associated with different histologic subtypes of endometrial cancer [4–9]. Previous investigations have been limited in providing molecular expression profiles specifically associated with early stage endometrial cancer [10–12]. Although mRNA microarray analysis is a powerful tool for assessment of gene expression, it is vital to obtain information at the protein level in order to take into account post-translational events.

The capability to precisely measure changes in the relative expression of numerous proteins simultaneously enables identification and facilitates our understanding of how the associated

pathways are linked in cellular networks. Proteomics aims to facilitate this process by applying newly developed methods and advanced analytical tools, such as mass spectrometry (MS), for investigation of the protein complement *en masse*. Proteomics represents the comprehensive study of cellular proteins and is aimed at analyzing their structure, function, modifications, expression, interactions and localization in complex biological systems. This global perspective provides the potential to observe changes within entire sets of cellular pathways and networks, thereby identifying those pathways key to the response of a cell to a changing microenvironment. Proteomics has greatly matured over the past decade from a demonstration science largely driven by analytical chemistry, to an applied science where the focus is increasingly toward answering biologically and pathophysiologically relevant questions. A great deal of this advancement has been ushered by increasingly robust technologies, such as protein/peptide separations and MS instrumentation, along with revolutions in bioinformatic methods for analyzing the high-dimensionality data. These novel analytical methods using tissue lysates offer a valuable opportunity to bolster our understanding of endometrial cancers and provide insight into the biology of early stage disease.

A high resolution MS-based proteomic analysis was conducted on proteins isolated from laser microdissected epithelial cells from 91 pathologically reviewed stage I endometrial cancer tissue samples (79 endometrioid and 12 serous) and 10 samples of normal endometrium from postmenopausal women. This analysis resulted in the identification of many differentially abundant proteins in stage I endometrial cancer patient tissues involved in novel pathways that likely play important supporting roles in the development and progression of early endometrial carcinogenesis.

Methods

Tissue samples

Fresh frozen endometrial cancer tissue specimens from 91 FIGO stage I endometrial patients collected either at Duke University Medical Center or by the Gynecologic Oncology Group were used for this analysis (79 endometrioid and 12 serous) [13]. The distribution by grade and stage are as follows: 9 IAG1, 14 IAG2, 7 IAG3, 14 IBG1, 12 IBG2, 12 IBG3, 7 ICG1, 10 ICG2, and 6 ICG3. All tissues were collected from patients follow informed consent under IRB-approved protocols at the corresponding institutions. Endometrial tissue specimens were harvested by pathologists at the time of surgery and each tumor was frozen until the time of the analysis. Hematoxylin and eosin (H&E) stained tissue specimens were evaluated by one of two board certified gynecologic pathologists to confirm the diagnosis. Ten age-matched normal endometrial samples from post-menopausal women undergoing hysterectomy for reasons other than abnormal uterine bleeding or malignancy were used to create a control sample set.

A Tissue Microarray (TMA) was constructed for validation using formalin-fixed, paraffin-embedded tissue samples (0.6 μ m) from the Armed Forces Institute of Pathology. Each case was represented in triplicate on the TMA. The tissue cores corresponded to the following tissue types: 145 endometrial carcinoma, 26 perimenopausal endometrium, and 24 postmenopausal endometrium. Three cases of endometrial cancer found to be duplicated on

the TMA were excluded leaving a total of 139 cases and 50 controls for analysis. Substage and grade were unknown for one patient. For the others, substages were 43 IA, 49 IB, and 46 IC; grades 57 G1, 29 G2 and 52 G3.

Tissue preparation for proteomics

Tissue samples were cut by microtome into 10 μ m sections and placed onto laser microdissection slides (Carl Zeiss MicroImaging). Cancer samples (~15 slides each) were laser microdissected to provide >95% purity of cancer and 2 control sets of normal endometrium were prepared. Ten control samples of normal endometrium from postmenopausal patients were collected by laser microdissection (~40 slides each) of endometrial glands and surrounding stroma. Cells were procured directly into microcentrifuge tubes containing 100 μ L of 0.2mM 3-[3-(1,1-bisalkoxyethyl)pyridin-1-yl]propane-1-sulfonate (PPS Silent Surfactant, Protein Discovery, Inc., Knoxville, TN), 50mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM DTT, and were stored at -80°C until digestion. Prior to proteomic analysis, samples were thawed, sonicated and digested with porcine sequencing grade trypsin (Promega) at a final concentration of 500 ng/50,000 cells. Following digestion, the PPS Silent Surfactant was cleaved by addition of 50 μ L of neat formic acid and incubated at 37°C for 2 h. The peptide digests were desalted by PepClean spin columns (Pierce) according to the manufacturer's instructions and dried by vacuum centrifugation.

Liquid chromatography tandem mass spectrometry

Tryptic peptide digests were resuspended in 30 μ L of 0.1% TFA, each of which were equally divided into two 15 μ L aliquots, each of which were independently analyzed in duplicate by LC-MS/MS with an LTQ-FT (ThermoFisher Scientific, Inc, San Jose CA) or an LTQ-Orbitrap (ThermoFisher Scientific Inc.) MS, for a total of 202 LTQ-Orbitrap and 202 LTQ-FT analyses. Peptide separations were conducted online with MS by nanoflow liquid chromatography. After injection of the tissue proteome digest, the column was washed with 98% mobile phase A (0.1% formic acid in water) for 30 min and peptides were eluted by development of a linear gradient of 2% mobile phase B (0.1% formic acid in acetonitrile) to 42% mobile phase B in 140 min, then to 98% B in an additional 20 min, all at a constant flow rate of 250 nL/min. The LTQ-Orbitrap MS was operated in a data-dependent MS/MS mode in which each high resolution broadband MS scan ($R=60,000$ at m/z 400, precursor ion selection scan range of m/z 350–1800) was followed by seven MS/MS scans in the linear ion trap where the seven most abundant peptide molecular ions dynamically determined from the MS scan were selected for tandem MS using a relative collision-induced dissociation (CID) energy of 30%. The LTQ-FT MS was operated in a data-dependent MS/MS mode in which each high resolution broadband MS scan ($R=50,000$ at m/z 400, precursor ion selection scan range of m/z 50–1700) was followed by three MS/MS scans in the linear ion trap where the three most abundant peptide molecular ions dynamically determined from the MS scan were selected for tandem MS using a relative collision-induced dissociation (CID) energy of 30%. Dynamic exclusion was utilized to minimize redundant selection of peptides for CID.

Bioinformatic and biostatistical analysis

Tandem mass spectra were searched against the UniProt human proteome database (<http://www.expasy.org>, 05/07 version) using SEQUEST (ThermoFisher Scientific Inc.). Peptides were considered legitimately identified if they achieved specific charge state and proteolytic cleavage-dependent cross-correlation (Xcorr) scores of 1.9 for $[M+H]^1+$, 2.2 for $[M+2H]^2+$, and 3.5 for $[M+3H]^3+$, and a minimum delta correlation score (Cn) of 0.08. These criteria resulted in a false discovery rate (FDR) of 1.4% for all peptides identified as determined by searching the entire data set against a decoy human database where the protein sequences were reversed (Elias, J.E. and S.P. Gygi (2007). "Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry." *Nat Methods* 4(3): 207–214.). A quantitative estimate of the relative abundance of proteins was obtained by comparison of the number of sequenced peptides per protein (e.g. spectral counting), such that if a protein is present in greater abundance it will be identified through a relatively greater number of peptide MS/MS sequencing events in one sample versus another [14,15]. Total protein spectral count values were arrived at by summing the spectral counts from the two replicate LC-MS/MS analyses from the two MS instrument platforms.

Analysis was restricted to the proteins with total spectral count >10, and sample spectral counts were normalized by the total spectral count value per tissue sample, which ranged over a factor of 3.12. (Note that results in Fig. 1 are not normalized.) We performed four comparisons: normal endometrium control versus all endometrial cancers, normal versus endometrioid cancer, normal versus serous cancer, and serous versus endometrioid endometrial cancer. Each comparison proceeded as follows. Wilcoxon two-sided two-sample tests produced a collection of P values. From this collection of P values, Q values were estimated [16] using the R package "qvalue". The Q value for a protein estimates the proportion of false positives among the subset of proteins with P values at least as small as the P value for this protein. The Q value method accounts for multiple comparisons and consistently estimates the Bayesian posterior probability of false positives.

Multiplying each Q value by the protein's ranking estimates the expected number of false discoveries if that protein's P-value is used as a cutoff. For each comparison (normal versus cancer, etc.), the cutoff was chosen so that the expected number of false discoveries was less than one.

Protein fold changes were used as the input to the IPA software (Ingenuity Systems, Redwood City, CA) for pathway analysis.

Western blot analysis

Four proteins were selected for verification/validation by western blot analysis and included annexin A1 (ANXA1) and A2 (ANXA2), lactate dehydrogenase (LDH) and peroxiredoxin 3 (PRDX3), along with glyceraldehyde phosphate dehydrogenase (GAPDH) as a loading control. Laser microdissected cells from fresh-frozen tissue samples were resuspended in SDS-PAGE loading buffer (Invitrogen) to a final concentration of 2000 cells/ μ L and boiled for 5 min. Approximately 25,000 cells (12.5 μ L) per sample were resolved via 1D SDS-PAGE and transferred to a PVDF membrane at a constant 30 V/160 mA for 2 h. Blots were

blocked for 2 h at ambient temperature in 1X blotto (5% dehydrated milk, 0.1% Tween-20 and 1X TBS) followed by incubation in primary antibody diluted at 1:1000 in 1X blotto overnight at 4 °C. Blots were washed in 1X TBST (1X TBS, 0.1% Tween-20) followed by incubation in secondary antibody for 1 h at ambient temperature at a condition-specific concentration (detailed below). Blots were washed again in 1X TBST followed by incubation in chemiluminescent substrate (SuperSignal West Pico, Pierce) for 5 min. Antibody and secondary antibody (2nd) conditions utilized were as follows: goat anti-rabbit IgGHRP (Abcam: ab6721), goat anti-mouse IgG-HRP (Pierce), anti-human GAPDH (Santa Cruz: sc-47724; 2nd at 1:100 K), anti-human annexin A1 (Cell Signaling: 3299; 2nd at 1:10 K), anti-Annexin A2 (Abcam: ab54771; 2nd: 1:100 K), anti-human lactate dehydrogenase (Abcam: ab55433; 2nd: 1:50 K) and anti-human peroxiredoxin 3 (Abcam: ab16753; 2nd at 1:50 K). Densitometry data were obtained from western blot films scanned on a BioSpectrum 2D Imaging System (UVP, Upland, CA) and analyzed utilizing VisionWorksLS Image Acquisition and Analysis software (UVP). Briefly, virtual boxes were drawn around each signal band on the western blot as well as around adjacent space not containing a signal to facilitate blot background subtraction. Values were calculated for the pixel intensities present within each boxed area and non-signal band values were subtracted from the signal containing band values to obtain a measurement of band/pixel intensity devoid of blot background signal. Reported results represent band densities subtracted from adjacent background densities normalized to corresponding GAPDH expression for each sample to achieve relative fold-change values.

Tissue microarray analysis

Two proteins were selected for independent validation using the TMA. The tissue microarrays were cut into 4 µm sections and mounted on positively charged glass slides. After deparaffinization and antigen retrieval (1 mM EDTA buffer) using standard methods, the slides were incubated with the primary antibody (ANXA2 [Abcam, 1:400 dilution] and PRDX1 [Abcam, 1:500 dilution]) overnight at room temperature. Following washing in buffers, the slides were incubated with 0.3% hydrogen peroxide in TBS for 10 min to block endogenous peroxidase activity. Dako Dual linker was applied to the slides prior to further rinsing in TBS to reduce background staining. The slides underwent a series of dehydration steps with 95% and 100% ethanol, and were then mounted with coverslips. Positive and negative controls were used for each antibody; the positive control for ANXA2 was a section of normal tonsil and normal liver was used for PRDX1. Negative controls were incubated with an anti-rabbit/anti-mouse serum mixture without primary antibody.

The intensity of staining was evaluated in endometrial carcinomas, and accompanying controls by a board certified pathologist. For all cases and controls, the intensity of the staining was evaluated visually and graded. A numerical score was assigned to each spot on the TMA with a score of 0 for negative, 1 for weak, 2 for moderate, and 3 for strong staining. Using this scoring schema, a mean score was calculated for each sample present in triplicate. In evaluating cases where no tissue was present in one of the 3 spots, a mean score was calculated from the intensity scores of the 2 spots. Differences in staining were tested with ANOVA, and two-sided, two-sample Wilcoxon and Student's t-tests.

Results

Endometrial tissue proteomic analysis

Despite the analytical advantages demonstrated by the use of laser microdissection for cell-type specific genomic analysis, similarly productive applications of corollary proteomic analysis of microdissected cell populations have been limited. Obtaining high quality proteomic results using nanoflow liquid chromatography (nanoLC) coupled online with tandem mass spectrometry (MS/MS), a key analytical method of proteomics, has typically required more cells than can readily be collected by laser microdissection. Additionally, when time or tissue sample sizes are limiting, as is often the case with fresh or frozen clinical specimens, research strategies that rely on pooling cells captured from multiple sections are unfeasible.

To increase protein recovery/yield and MS signal intensity from small populations of cells, we have developed a single-tube protocol for preparing proteins from laser microdissected cells. The single-tube protocol relies on the special characteristics of a novel MS-compatible surfactant (PPS Silent Surfactant) that acts as an aggressive detergent for cell disruption and protein solubilization, while eliminating buffer changes and sample cleanup steps that otherwise reduce protein yield. PPS Silent Surfactant (3-[3-(1,1-bisalkoxyethyl)pyridin-1-yl] propane-1-sulfonate) is a cleavable surfactant which rapidly hydrolyzes at low pH into cleavage products that have no remaining surfactant properties and do not interfere with downstream MS analysis. The new protocol has been applied to a proteomic investigation to identify differentially abundant proteins in early stage endometrial cancer utilizing cells acquired by laser microdissection from endometrial tissue samples.

Samples were aliquoted into two equal volumes and each lyophilized to dryness. Each aliquot (30,000 cell equivalents) was analyzed in duplicate by nanoLC coupled online with both an FTICR or an Orbitrap MS and total proteins and differential abundances were determined by spectral counting [14,15]. Total protein spectral count values were arrived at by summing the spectral counts from the two replicate LC-MS/MS analyses from the two MS instrument platforms.

Wilcoxon two-sample tests were performed on the total protein spectral counts normalized by the total spectral count value per tissue sample for each of the three analyses: normal endometrium control versus either endometrioid, serous only or all endometrial cancers, as well as serous versus endometrioid endometrial cancer. Proteins with greater than 10 total spectral counts were selected for analysis and ranked by Q values cancer [16], which estimate the positive false discovery rate. The cutoff was chosen so that the expected number of false discoveries was less than one. The cutoff Q value was 0.0047 for endometrial cancer versus normal endometrium (209 proteins), 0.0042 for endometrioid endometrial cancer versus normal endometrium (217 proteins), and 0.0053 for serious endometrial cancer versus normal endometrium (186 proteins). The complete lists of differentially expressed proteins for each of the protein expression analyses are provided in Supplemental File 1. The distribution of select differentially expressed proteins from the cancer versus normal endometrium comparison (ANXA1, ANXA2, PRDX3, PRDX4, PRDX5, PRDX6, and COX2) are shown in Fig. 1.

Validation

In an independent sample set of frozen tissues, two post-menopausal endometrium specimens and twelve endometrioid endometrial cancer tissue samples were evaluated by western blot analysis to verify the spectral count-derived differential expression of LDH, annexin A1 (ANXA1) and A2 (ANXA2) and peroxiredoxin 3 (PRDX3) (Fig. 2A). The western blot analyses (Fig. 2B and Table 1) for each of these four proteins unambiguously agree with the spectral count-derived differential abundance measures for these proteins as elevated in abundance in endometrial cancer tissue.

Additionally, we constructed a tissue microarray using a naïve and independent tissue sample set containing tissue specimens representative of the endometrium from perimenopausal and post-menopausal women and stage I endometrial cancers. Immunohistochemical staining in the tissue microarray strongly confirmed the increased abundance of ANXA2 (Fig. 3A) and PRDX1 (Fig. 3B) in endometrial cancer tissue compared to normal endometrium. Strong and diffuse cytoplasmic staining (staining intensity 3+) with some non-specific background stromal staining was noted for ANXA2 antibody in a section of endometrial carcinoma. No nuclear staining was noted (Fig. 3A1). In contrast, negative staining of ANXA2 was noted in the normal endometrium (Fig. 3A2). Strong and diffuse cytoplasmic with no nuclear staining of PRDX1 was noted in a representative endometrial carcinoma case (Fig. 3B1). A normal endometrium control showed negative staining from the same tissue microarray block (Fig. 3B2).

We compared PRDX1 staining between cancer specimens and those of perimenopausal and postmenopausal subjects without cancer. There was no evidence that perimenopausal and postmenopausal results differed ($p>0.6$ Wilcoxon and Student's t -test), so they were pooled. Levels of PRDX1 were higher in the cancer specimens compared to the normal controls ($p=0.00749$ Wilcoxon, $p=0.00965$ Student's t -test). The means were 1.82 and 1.46 respectively (SEM=0.046, 0.120). For ANXA2 the means ($\pm$ SEM) for cancer, perimenopausal normal and postmenopausal normal results were 2.31 ± 0.065 , 1.99 ± 0.17 , 1.55 ± 0.145 , respectively ($p=2.3 \times 10^{-5}$, ANOVA). The difference between perimenopausal and postmenopausal results was equivocal ($p=0.028$ Wilcoxon, 0.06 Student's t -test). Comparisons between cancer and perimenopausal normal tissues were highly significant ($p=1.22 \times 10^{-6}$ Wilcoxon, $p=2.83 \times 10^{-5}$ Student's t -test); the differences were similarly significant if the postmenopausal samples and perimenopausal samples were combined (Fig. 3 C). Data collected from the TMA and the Western analysis correlated with the spectral count-derived increased abundance of ANXA2 and PRDX1 in endometrial cancer tissue.

Network analysis

A protein network analysis of the proteomic data was undertaken using Ingenuity Pathway analysis software to identify significant interaction relationships among the differentially abundant proteins in manifold signaling and interaction pathways. Using the top 175 ranked protein cancers compared to normals, several statistically significant networks were identified. Of these the top 10 networks had IPA scores of 16 or higher (Supplementary File 2). The most statistically significant network contained 28 interacting proteins from our proteomic analysis. This analysis revealed pathways associated with mitochondrial

dysfunction and involved multiple members of the peroxiredoxin family (Fig. 4), including PRDX3, which have been previously described in association with other types of solid tumors. In addition, pathways associated with oxidative phosphorylation were also identified as significantly deregulated in stage I endometrial cancer tissue. A similar analysis was conducted with endometrioid versus normal and serous versus normals. These networks are included in Supplementary File 2.

Discussion

Our group elected to analyze tissues samples in duplicate using two MS platforms (LTQ-FT and LTQ-Orbitrap) to conduct a global differential proteomic investigation of stage I endometrial cancer compared to normal endometrium. De Douza et al. [17] have used multi-dimensional liquid chromatography and tandem mass spectrometry in comparison of twenty Type I and Type II endometrial cancers with normal endometrium from twenty premenopausal women (10 secretory and 10 proliferative). Similarly, Byrjalsen et al. [18] used two-dimensional gene analysis coupled with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to evaluate 5 endometrial cancers, 5 endometrial hyperplasias and 6 normal endometrium (5 of which were proliferative). The small numbers of tissue samples and less sensitive methods of proteomic analysis utilized likely resulted in the limited identification of differentially expressed proteins in these reports.

For these reasons, along with fundamental differences in sample selection and preparation, it is difficult to render an accurate comparison of these previous data to our present study. Of note, we identified multiple proteins present at increased relative abundance in stage I endometrial cancer compared to normal tissue indicative of mitochondrial dysfunction. Mitochondrial dysfunction is becoming a recognized hallmark of carcinogenesis with a number of reports and mathematical models demonstrating significant increases in acidosis and hypoxia in the tissue microenvironment even at the earliest stages of carcinomas in situ [19]. The ability of tumor cells to adapt to and withstand increasing levels of hypoxia and acidosis is required for tumor progression and proliferation. It is well established that cells exhibit increased levels of glucose utilization for glycolysis under hypoxic conditions (the Pasteur effect) and that glycolysis is generally constitutively upregulated in cancer cells (the Warburg effect), enabling tumors cells to produce energy in an oxygen independent manner. Tumor cells therefore must adapt to accommodate the effects (namely generation of reactive oxygen species) of an ever increasing hypoxic and acidic microenvironment, and data presented here specifically support the notion that endometrial cancer cells exhibit these adaptive response mechanisms at the earliest stages of disease, namely stage I, an observation that has not been described to date from patient tissue samples. A number of differentially abundant proteins support this notion, including multiple members of the peroxiredoxin family (i.e. PRDX1, PRDX3, PRDX4, PRDX5, and PRDX6) which were found to be overexpressed in our proteomic analysis. The peroxiredoxin family of enzymes is involved in scavenging of peroxides, and in cancer cells confer a pro-survival phenotype and support tumor maintenance [20]. While peroxiredoxins do not suppress cell proliferation typical of tumor suppressor genes, loss of PRDX1 can result in tumor formation [21]. In addition, overexpression of specific peroxiredoxins can promote tumor survival via

protection from oxidative stress-induced apoptosis [22]. Ablation of PRDX3 has been shown to increase sensitivity of cancer cells to the effects of oxidation stress [23,24]. Furthermore, overexpression of PRDX5 has been associated with shorter survival in breast cancer patients [25] while enhanced expression of PRDX1 and PRDX6 have been associated with development, recurrence and progression of bladder cancer [26,27]. Although there have not been any previous reports highlighting peroxiredoxin overexpression in endometrial cancer, loss of the PTEN tumor suppressor gene (a common mutational event associated with endometrial cancer) in mouse embryonic fibroblasts has been associated with peroxiredoxin dysregulation [28].

Prohibitin 2 (PHB2) was identified in our investigation as overexpressed in endometrial cancer tissue. PHB2, which is predominantly localized to the mitochondrion, is important in regulation of apoptosis as well as maintenance of mitochondrial morphology [29]. In the presence of estradiol (E2) and estrogen receptor (ER), PHB2 translocates to the nucleus where it acts to repress ER-dependent transcription [30]. Increased expression of PHB2 has been shown to correlate directly with ER levels, and inversely with tumor grade, in breast cancer patients [31]. The identification of an increased abundance of PHB2 in endometrial cancer tissue as compared to normal endometrium may reflect an estrogen-driven microenvironment associated with endometrioid endometrial cancer. Notably, another proteomic investigation of five cancers and five hyperplasias also identified prohibitin as increased in abundance in endometrial cancer suggesting that deregulation of this protein may accompany transformation, although it may not represent a biomarker for precancerous endometrial lesions [18].

Interestingly, thirteen of the top 215 proteins that were identified as differentially abundant in endometrial cancer compared to normal endometrium were ribosomal proteins (RS3, RS9, RS14, RS18, RLA1, RLA2, RL8, RL11, RL22, RL18, RL24, RL10A and RL27A) and have not been previously described as associated with endometrial cancer. Previous investigations have reported differential expression of ribosomal proteins in association with breast [32], liver [33], and gastric [34] carcinoma compared to corresponding normal epithelial controls. Recent advances in ribosomal research have indicated that ribosomal proteins have a variety of extraribosomal functions (independent of protein biosynthesis) such as replication, DNA repair, inflammation, apoptosis, and scavenging of reactive oxygen species [35]. Comparative analysis of different epithelial cancers indicates that overexpression of specific ribosomal proteins may be uniquely associated with specific tumor types and not necessarily a byproduct of overall proliferation among tumors. Among the ribosomal proteins that we observed in association with stage I endometrial cancers, RS18 and RL18 have been previously reported in association with colon cancer [36] and RS14 and RS18 have been reported in prostate cancer [37].

Proteomic analysis revealed that several members of the annexin family were differentially expressed. The annexin family is a large family of proteins that bind (i.e. annex) to cellular membranes in a calcium dependent manner. There are 13 annexin members each of which share a common repetitive 70 amino acid motif, along with a variable amino terminal that mediates their specific function. These molecules, also known as lipocortins, mimic several glucocorticoid functions particularly as it pertains to anti-inflammatory properties [38].

ANXA1 and ANXA2 were found to be significantly overexpressed ($p < 0.05$) in both endometrioid and serous histologic types when compared to normal controls. Annexin AI has been implicated in a variety of glucocorticoid functions including cellular proliferation and migration [39] and its overexpression has been reported for multiple cancers including pancreatic [40], breast [41], and oral carcinoma [42], and among patients with esophageal carcinoma is associated with a higher rate of recurrence and worse survival compared to patients with lower ANXA1 expression levels [43]. These data also point to a significant increase in abundance of LDH in early stage endometrial cancer. Tumors with enhanced LDH have an apparent lack of lymphocytic response at the tumor edge, suggesting that lactate production may have a protective role from immuno-surveillance [44]. Recent clinical trials in colon cancer have demonstrated a survival benefit to the addition of VEGF agents to adjunctive chemotherapy in the treatment of colon cancer patients with elevated LDH [45]. Together our findings suggest that endometrial cancers may have differential changes in gene and protein expression that promote a protective anti-inflammatory microenvironment supportive of tumor survival.

Network analysis revealed significant interaction between pathways of differentially expressed families of molecules including differentially expressed proteins associated with mitochondrial dysfunction (PRDX3, PRDX4, PRDX5, PRDX6, Jnk, etc.) as well as processes that are involved in altered oxidative phosphorylation (COX2, COX6C, COX7A2, etc.). In addition, nuclear factor kappa beta (NF κ B), which regulates genes that encode, cytokines, cytokine receptors, and cell adhesion molecules associated with inflammatory processes, was found to have relationships with LDHA and LDHB, anti-inflammatory molecules found to be upregulated in our proteomic analysis.

Conclusions

Although the present study resulted in the identification of differentially expressed proteins known to be associated with endometrial cancer, several dysregulated signaling and metabolic networks not previously described in association with endometrial cancer were also identified. These data demonstrate that MS-based proteomic analysis of endometrial cancer cells laser microdissected from patient tissue samples collected at the time of surgical staging offers an innovative approach toward identification of dysregulated protein networks and candidate biomarkers associated with the early stages of carcinogenesis in this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Disclosures

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Fig 1.

Distribution of select differentially expressed proteins from the cancer versus normal endometrium comparison (ANXA1, ANXA2, PRDX1, PRDX3, PRDX5, PRDX6, COX2, COX6C, prohibitin 2, RL18 and RS18). P-values are shown for the Wilcoxon two-sample test (W) and for the Fisher exact test on the 2 by 2 presence/absence table (F). Each box extends from first to third quartile. Dark horizontal line is the median. The whiskers extend to the most extreme data point that is no more than 1.5 times the interquartile range from the box.

Fig. 2.

A) Western blot analysis used to provide internal validation of normal postmenopausal endometrium and endometrial cancer tissue. B) Mean densitometric abundance levels of lactate dehydrogenase, annexin A1 and A2 and peroxiredoxin 3 derived from Western blot analyses of two normal postmenopausal endometrium and twelve endometrial cancer tissue samples. All values are normalized to GAPDH levels determined for each of these samples to control for loading.

Fig. 3.

Representative immunohistochemical staining of ANXA2 (A) and PRDX1 (B) in cancer (left panels, A1 and B1) and normal postmenopausal endometrium (right panels, A2 and B2) in a tissue microarray. (C): Boxplots for tissue microarray blinded pathologic assessments for annexin A2 (ANXA2) and peroxiredoxin (PRDX1), taking on values 0, 1, 2, and 3. The boxes extend to first and third quartiles, thick lines are at the median, and whiskers extend to 1.5× width of box or to the range of the data. The P-value for ANXA2 is from analysis of variance. The P-value indicated by (A) is from a Wilcoxon test normalizing for total sample spectral count.

Fig. 4.

Ingenuity Pathway analysis of endometrial cancers (serous plus endometrioid) versus normal “epithelium plus stroma” controls revealed 8 networks that were statistically significant based on the number of differentially expressed focus molecules. Interactions associated with Network 1 revealed processes associated with mitochondrial dysfunction and oxidative phosphorylation including peroxiredoxin family members (PRDX1, PRDX3, PRDX4, PRDX5, and PRDX6) and cyclooxygenase 2 (COX2) as well as NRF2-mediated oxidative stress response including peroxidase, PRDX1, GSTK1, glutathione transferase, and GSTP1.

Table 1

Densitometric results from Western blot validation of lactate dehydrogenase, annexins A1 and A2 and peroxiredoxin 3.

	N1 ^a	N2	T1 ^b	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
LDH ^c	-0.03 ^e	-0.05	-0.01	0.6	0.72	0.09	0.02	0.99	2.25	3.9	2.44	2.25	1.61	0.32
Annexin A1	-0.03	-0.02	-0.01	0.34	0.26	0	0.33	1.71	0.99	-0.03	0.01	0.73	1.42	0.06
Annexin A2	-0.01	-0.01	-0.01	0.08	0.13	0.32	0.17	1.52	1.65	1.63	0.78	1.96	2.19	3.08
PRDX3 ^d	-0.04	-0.02	0.07	1.23	0.88	0.8	0.32	0.66	0.6	-0.03	-0.01	0.82	1.21	0

^aN – Normal, non-cancer tissue.

^bT – Endometrial tumor tissue.

^cLDH – Lactate dehydrogenase.

^dPRDX3 – Peroxiredoxin 3.

^eValues normalized to GAPDH.