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Novel Surgical Approaches for Sampling the Ovarian Surface Epithelium and Proximal Fluid Proteome

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Abstract

The pathogenesis of ovarian, fallopian tube, and peritoneal cancers has been difficult to elucidate despite intense effort. Recently, though, the care of women felt to be at high risk due to a strong family history of breast and/or ovarian cancer or a known germline *BRCA1* or *BRCA2* mutation has provided potential insight into the development of these malignancies. Risk-reducing surgical removal of the fallopian tubes and ovaries, called risk-reducing bilateral salpingo-oopherectomy (RRBSO), is commonly performed as a laparoscopic procedure to minimize recovery time. We describe here an optimized surgical sampling workflow for analyzing the proteomes of peritoneal, fallopian tube, and ovarian surface epithelial (OSE) specimens collected at the time of laparoscopic RRBSO, a technique which has not been described previously. This methodology presents a unique opportunity for closer examination of the proteomic alterations in the tissues at risk for malignant transformation in women with an inherited susceptibility to ovarian, fallopian tube, and peritoneal cancer development.

Keywords

ovarian cancer; ovarian surface epithelium; fallopian tube; proteomics; mass spectrometry

Introduction

Epithelial ovarian cancer (EOC) is the most lethal of the gynecologic cancers and is responsible for almost 16000 deaths each year in the United States.[1] Fallopian tube and primary peritoneal cancers are thought to be related to ovarian cancer given the similarity in their histology, clinical behavior, and response to treatment. The pathogenesis of all three cancers has been difficult to elucidate despite intense effort. Recently, though, the care of women felt to be at high risk due to a strong family history of breast and/or ovarian cancer

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or a known germline *BRCA1* or *BRCA2* mutation has provided potential insight into the development of these malignancies.

The discovery of the *BRCA1* and *BRCA2* genes led to an increased awareness of hereditary breast and ovarian cancer and has caused more women to choose risk-reducing surgery, generally entailing bilateral salpingo-oophorectomy (BSO) to remove the fallopian tubes and ovaries with or without hysterectomy. Cancer prevalence at the time of prophylactic surgery has ranged from 1.1 to 25%, with the largest prospective study to date reporting a prevalence of 2.2%.[2,3] One of the intriguing findings from these studies is the unexpectedly high proportion of fallopian tube cancers seen (22 out of 50 cancers in *BRCA* mutation carriers in one recent review).[2] This has led to intense focus on the fallopian tube as the possible source of ovarian cancer. By convention, cancer is deemed ovarian in origin when the ovaries and fallopian tubes are involved extensively in tumor; the fallopian tube is considered the origin only if the ovaries are either only superficially involved or completely spared. Since approximately 75% of ovarian cancer cases are diagnosed at stage III (once disease has already spread from the pelvis to the upper abdomen), it is possible that a primary tumor in the fallopian tube has been lost in the bulky adnexal disease in some "ovarian" cancer cases. Indeed, a recent series of 55 serous carcinomas involving the pelvis found that while 43, 7, and 5 cases were considered "ovarian," "peritoneal," and "tubal" in origin, respectively, based on traditional pathologic criteria, 41 (75%) demonstrated evidence of tubal intraepithelial carcinoma (TIC). TIC may represent an intermediary step in the development of many serous gynecologic cancers, including those that traditionally have been thought to arise in the ovarian surface epithelium or peritoneum.[4]

Application of molecular methods to profile specimens collected from women with an inherited risk for EOC before they develop overt disease may allow for better insight into the pathogenesis of EOC. Prior attempts to utilize specimens from risk-reducing surgeries have faced several challenges. Most of these studies have focused on the ovaries alone, excluding the fallopian tubes and peritoneum from study.[5–10] This may be problematic if the disease process is truly a pelvic serous carcinoma, where the entire pelvic field is at risk, or alternatively if the OSE is not the origin of what is currently classified as ovarian cancer. Another issue is the type of samples used, such as fresh frozen ovarian tissue or cell lines cultured from OSE.[5–10] The OSE is a single cell layer that is often lost during manipulation, hence fresh frozen ovarian specimens contain little OSE and are predominantly stromal. Further, the gene expression profiles from OSE cells grown in cell culture differ markedly from uncultured OSE.[11] Finally, several analyses have relied on traditional pathologic evaluation of the OSE for changes such as inclusion cysts and/or immunohistochemical staining as possible indicators of malignant transformation.[5–8] In cases where advanced molecular profiling techniques have been utilized, all comparisons included OSE exposed to cell culture but contained no data from fresh, uncultured tissue.[9,10]

Risk-reducing BSO (RRBSO) is commonly performed as a laparoscopic procedure to minimize recovery time and presents a unique opportunity for closer examination of the tissues at risk for malignant transformation in women with increased genetic susceptibility to ovarian, fallopian tube and peritoneal cancer development. The collection of tissue specimens at the time of RRBSO is complicated by the need to maintain the integrity of the ovaries and fallopian tubes for clinical pathologic analysis. The current standard technique in high-risk women involves sectioning the entire ovary and fallopian tube to allow subcentimeter lesions to be identified.[12,13] Hence, specimen collection for research purposes must allow for standard pathologic processing, making removal of a portion of fallopian tube or ovary for research purposes medically and ethically unacceptable.

Proteomic evaluation of specimens collected from pelvic tissues at the time of laparoscopic surgery has not been described previously. The purpose of this study was to refine our protocol for acquiring ovarian surface epithelial cells and proximal fluids from the fallopian tubes and peritoneal cavity to enable a workflow for harvesting proteins from the environment where EOC arises. This report is the first description of a novel workflow for collecting surgical specimens in this setting suitable for mass spectrometry (MS)-based proteomics.

Materials and Methods

Patient Selection

Subjects were identified from the University of Pittsburgh Cancer Institute Cancer Family Registry, a database that enrolls women from 3 broad categories: those with *BRCA1* mutations, those with *BRCA2* mutations, and those with family histories of breast and/or ovarian cancer without a known mutation. For this pilot analysis, a convenience subset of individuals with a *BRCA2* mutation was selected; the *BRCA2* mutation category accumulated patients faster than the other 2 categories in our registry and, therefore, was picked to provide a relatively homogeneous initial group to evaluate. Patients provided informed consent according to a protocol approved by the University of Pittsburgh Institutional Review Board.

Surgical Specimen Collection

All subjects had blood samples collected preoperatively. Tissue was collected at the time of laparoscopic RRBSO with or without hysterectomy. RRBSO was performed according to established protocol.[12,13] The procedure included visual inspection of the peritoneal cavity, collection of a peritoneal lavage for cytology, and removal of both ovaries and fallopian tubes. Meticulous pathological processing of the surgical specimen was performed, with 2 mm serial sections through the entire adnexae.

Abdominal Entry

Open laparoscopy was performed by making a 10 mm incision at the inferior aspect of the umbilicus through which a 10 mm port was placed. Pneumoperitoneum was established with CO_2 to distend the abdomen and facilitate surgical exposure. Two 5 mm laparoscopic ports were placed in each lower quadrant under direct visualization.

Peritoneal Washings

Prior to the manipulation of the adnexae, the pelvic peritoneal cavity was irrigated with approximately 50 mL of Lactated Ringer solution using a laparoscopic suction-irrigator. From this aspirate, 5 mL were collected in a sterile container for research purposes while the remainder was submitted for routine cytologic analysis.

OSE Brushings

Bilateral OSE brushings were collected as soon as adequate exposure had been obtained and prior to manipulation of the adnexae. Brushings of OSE were collected separately from each ovary with a cytology brush originally developed for use during colonoscopy (Bard Interventional Products Division, Billerica, MA), stabilized through a laparoscopic open-port Kumar clamp (Figure 1). The cytology brush has a sheath that allows the brush to be retracted and protected after OSE collection until it is removed from the port. Each sterile cytobrush tip was placed in 1 mL of 50% acetonitrile/50 mM ammonium bicarbonate solution.

Fallopian Tube Lavage

Fallopian tube lavage was collected immediately after each adnexa was removed from the abdominal cavity by placing them in separate 10 mm endoscopic bags as soon as the blood supply was severed to minimize ischemic time. The fimbriated end of each fallopian tube was cannulated with a small-caliber, flexible plastic tube such as a 24-gauge angiocath and irrigated with 5 mL of normal saline that was collected as it drained back out of the fimbriae.

Specimen Processing

All specimens were processed within 30 min of collection of the fallopian tube lavage.

OSE Brushings

The cells were lysed by boiling for 10 min in 1 mL of 50% acetonitrile/50 mM ammonium bicarbonate. Samples were diluted 1:3 with 50 mM ammonium bicarbonate and concentrated 10-fold with Amicon 3000 Da molecular weight cutoff filters (MWCO) (Millipore) at 2000× *g* at 4 °C. Total protein was determined by the Bradford assay. Chicken ovalbumin (50 fmol/μg total protein) was added to each sample to serve as an internal digestion and loading control. Each sample was loaded on a 4–12% Bis-Tris gel (Invitrogen) with MES (1×) running buffer and electrophoresed for 10 min such that the global protein lysate remained unresolved in the stacking gel. The single gel band was excised and digested in-gel with trypsin. Peptides were extracted three times with 70% acetonitrile/5% formic acid, pooled and vacuum-dried.

Peritoneal Washings and Fallopian Tube Lavage

For each sample, 3 mL were collected and concentrated 5- to 10-fold with Amicon 3000 Da MWCO filters at 2000× *g* at 4 °C. Due to the large and inconsistent amount of albumin that was present, along with other highly abundant “serum” proteins, it was deemed necessary to conduct an immunodepletion step to enable observation of lower abundance proteins. Briefly, 250 μL neat fallopian tube lavage or peritoneal wash was mixed with 62.5 μL of 4× Multiple Affinity Removal System (provided by Agilent Technologies, Santa Clara, CA) sample loading buffer (Buffer A). The entire diluted sample was filtered through a 0.2 μm filter to remove particulates and depleted according to the manufacturer’s protocol. Depleted samples were buffer-exchanged into 100 mM ammonium bicarbonate, pH 8.2 by centrifugation through prerinsed 5 kDa MWCO concentrators (Millipore) and brought to a final volume of 100 μL with 100 mM ammonium bicarbonate. Protein concentration of all samples was determined by Bradford assay. Chicken ovalbumin was added to each sample at a concentration of 50 fmol/μg total protein and each sample was processed by stacking gel/in-gel digestion as described above.

LC-MS/MS Analysis

Sample digests were resuspended to a concentration of 0.5 mg/mL in 0.1% trifluoroacetic acid and analyzed in duplicate by nanoflow reversed-phase liquid chromatography (LC)-MS/MS using a nanoflow LC (Dionex Ultimate 3000, Dionex Corporation, Sunnyvale, CA) coupled online to a linear ion trap MS (LTQ-XL, ThermoFisher Scientific, San Jose, CA). Separation of the samples was performed using 100 μm inner diameter × 360 μm outer diameter ×20 cm-long fused silica capillary columns (Polymicro Technologies, Phoenix, AZ) packed in house with 5 μm, 300 Å pore size Jupiter C-18 stationary phase (Phenomenex, Torrance, CA). Following sample injection onto a C-18 trap column (Dionex), the column was washed for 3 min with mobile phase A (2% acetonitrile, 0.1% formic acid) at a flow rate of 30 μL/min. Peptide digests were eluted using a linear gradient of 0.33% mobile phase B (0.1% formic acid in acetonitrile)/minute for 130 min, then to 95%

B in an additional 15 min, all at a constant flow rate of 200 nL/min. Column washing was performed at 95% B for 15 min for all analyses, after which the column was re-equilibrated in mobile phase A prior to subsequent injections.

The MS was operated in a data-dependent MS/MS mode in which each full MS scan (precursor ion selection scan range of *m*/*z* 350–1800) was followed by 7 MS/MS scans where the 7 most abundant peptide molecular ions dynamically determined from the MS scan were selected for tandem MS using a relative CID energy of 30%. Dynamic exclusion was utilized to minimize redundant selection of peptides for CID.

Bioinformatic Analysis

Tandem mass spectra were searched against the UniProt human protein database (11/09 release) from the European Bioinformatics Institute (http://www.ebi-.ac.uk/integr8), using SEQUEST (ThermoFisher Scientific). Additionally, peptides were searched for methionine oxidation with a mass addition of 15.9949 Da. Peptides were considered legitimately identified if they met specific charge state and proteolytic cleavage-dependent cross correlation 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 of 0.08. A false peptide discovery rate of 1.3% was determined by searching the primary tandem MS data using the same criteria against a decoy database wherein the protein sequences are reversed.[14] Results were further filtered using software developed in-house, and differences in protein abundance between the samples were derived by summing the total CID events that resulted in a positively identified peptide for a given protein accession across all samples (e.g., spectral counting).[15] The spectral count data were normalized for each protein accession by calculating the percent contribution of the spectral count values for each protein accession against the total number of peptides identified within a given sample.[1]

Results

Patient Population

For this analysis of our surgical collection technique, we selected a convenience sample of 6 patients who carry *BRCA2* mutations among the initial women undergoing RRBSO (specific mutations noted in Table 1). We sought to limit the possible heterogeneity present among the different clinical diagnoses represented (*BRCA1* mutation, *BRCA2* mutation, family history without a known mutation) in the pilot project. Patient demographics are shown in Table 1. The mean age was 45 years. All patients had a family history of breast cancer, while 2 patients had a personal history of breast cancer. Three patients had preoperative CA-125 levels collected which were all normal. Two patients underwent BSO only, while all other patients underwent hysterectomy and BSO. All pathology was benign.

Surgical Sampling Technique

Some patients were not able to undergo complete collection due to surgical absence of portions of the adnexae (i.e., prior salpingectomy or salpingo-oophorectomy) or distortion of the adnexal anatomy by disease process (i.e., scarring of the fallopian tubes from prior infection). One patient did not have left OSE collected due to a prior left oophorectomy, whereas 1 patient did not have right tubal lavage performed due to scarring of the tube.

Through refinement of the surgical specimen protocol, optimal collection of peritoneal samples was obtained though small volume irrigation (50 mL) of the pelvic peritoneal cavity prior to manipulation of the adnexae. Initial attempts to collect washings from the fallopian tubes *in situ* were not consistently successful due to difficulty cannulating the fallopian tube and retrieving fluid after it was instilled into the tube. We therefore collected the fallopian

tube lavage immediately after each adnexa was removed from the abdominal cavity to minimize ischemic time. Because the fallopian tubes are highly vascular structures that bleed easily, gentle handling was necessary during the cannulation and irrigation process. However, even with such care taken, some specimens bled during the excision or cannulation process, causing them to be excluded at the time of initial specimen processing as samples with visible hemolysis are considered inadequate for comparative analysis in proteomics.[17] For OSE collection, although the cytology brush can be introduced by itself through the port, we found that stabilizing it through a laparoscopic open-port Kumar clamp allowed for easier specimen collection since the cytobrush is quite flexible and otherwise can be difficult to position (Figure 1).

Protein Identification and Analysis

This workflow enabled the identification of a total of 265, 328, and 241 different proteins by 2 or more unique peptides each from the any of the OSE, fallopian tube and peritoneum samples, respectively, including MUC-16 (CA-125), multiple kallikrein protein family isoforms and BRCA2 (Supplemental Table 1, Supporting Information).

Comparison of Sample Types

Fallopian tube and OSE specimens were collected bilaterally, with the number of proteins identified from each side in individual patients shown in Table 2. The number of proteins identified on both the right and left side in individual patients ranged from 16–33% for OSE and 31–36% for fallopian tube mucosa. Figure 2 compares the identified proteins in the 3 sample types collected in any of the patients. A total of 77 common proteins were identified in the pooled peritoneum, OSE, and fallopian tube specimens; that is, among the 6 patients, at least 1 patient had those proteins present in her peritoneum, OSE, or fallopian tube specimens.

The 3 sample types were further analyzed for characteristics of the identified proteins. Table 3 describes the subcellular location of the proteins present in each of the sample types. Extracellular, membrane and cytoplasm were the most frequent subcellular locations for all 3 sample types with extracellular the predominant cellular compartment. The biological functions of the proteins in each sample type are shown in Table 4. Metabolism is the most common function among the 3 sample types; in addition, many of the identified proteins are involved in acute phase signaling and complement pathways.

Comparison Across Individual Patients

A total of 37 common proteins were identified in at least 1 specimen from all 6 patients. Many of these proteins are involved in cell signaling and interaction, cell growth and proliferation, cellular assembly and DNA replication and repair. In addition, 14 proteins are involved in acute phase response signaling. A total of 23 of the common proteins identified have been shown to be involved in cancer pathways. Three protein network associations were identified among these common proteins: genetic disorders, cell movement and cell-to-cell signaling and interaction, and tissue morphology. A total of 23 proteins (62%) are involved with the genetic disorders pathway.

Discussion

The use of mass spectrometry and protein network database algorithms to evaluate proteomic patterns associated with pathology or risk of disease has garnered a great deal of interest due to increased analytical sensitivity, accuracy, throughput, and protein database annotation. In this pilot study, we defined a high-throughput workflow for analyzing the proteomes of peritoneal, fallopian tube, and OSE specimens collected at the time of

laparoscopic RRBSO, a technique which has not been described previously. This methodology presents a unique opportunity for closer examination of the tissues at risk for malignant transformation in women with an inherited susceptibility to ovarian, fallopian tube and peritoneal cancer development.

A global discovery-driven proteomic analysis of these specimens identified a consistently large number of proteins. Approximately one-third of the proteins identified were present in both sides of the bilateral specimen collections from the fallopian tube and OSE. Since it is unclear whether the entire OSE and/or fallopian tube are at risk for developing hereditary EOC as a field effect or whether focal lesions are the source of tumorigenesis, further analysis of the biological similarities and differences between these bilateral specimens will be critical.

In addition, as our registry enrolls more women undergoing RRBSO, the likelihood of identifying lesions such as TIC and occult invasive cancer will increase. Both lesions are currently felt to be present in approximately 1–10% of the RRBSO specimens. This specimen collection and analysis protocol should enable an improved understanding of the process of malignant transformation and facilitate identification of bio-markers of precancerous and cancerous changes in the population at high risk for ovarian, fallopian tube, and peritoneal cancer due to the presence of a deleterious *BRCA* mutation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BSO	bilateral salpingo-oopherectomy
RRB-SO	risk-reducing BSO
EOC	epithelial ovarian cancer
OSE	ovarian surface epithelium
TIC	tubal intraepithelial carcinoma
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MWCO	molecular weight cutoff
BCA	bichinchoninic acid
DTT	dithiothreitol
LIT	linear ion trap
CID	collision-induced dissociateion
FDR	false discovery rate
IPA	Ingenuity pathway analysis

OD	optical density
H&E	hematoxylin and eosin
1D SDS-PAGE	one-dimensional sodium-dodecyl-sulfate polyacrylamide gel electrophoresis

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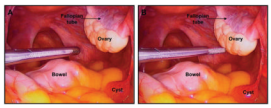
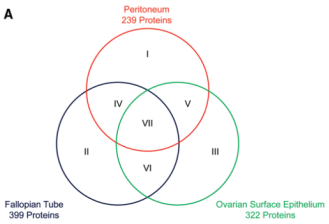


Figure 1.
OSE brushing collection with cytobrush and open-port Kumar clamp. (A) Kumar clamp and cytobrush are inserted into the abdominal cavity. A benign cyst is visible on the far right dangling from the fallopian tube. (B) Cytobrush is advanced from the protective sheath. The Kumar clamp stabilizes the ovary and cytobrush while the cytobrush is used to scrape the ovarian surface to collect OSE.

A



B

Region	Sample	Proteins Identified
I	P	80
II	FT	174
III	OSE	155
IV	P:FT	70
V	P:OSE	12
VI	FT:OSE	78
VII	P:FT:OSE	77
Total		646

Figure 2.

(A) Venn diagram depicting the total number of proteins and overlap of proteins identified for each sample type in fallopian tube (FT), peritoneum (P), and ovarian surface epithelium (OSE). (B) Numbers of proteins identified in each region of the Venn diagram for each sample type in fallopian tube (FT), peritoneum (P), and ovarian surface epithelium (OSE).

Table 1

Patient Demographics[a]

ID	age	race	parity	history of breast cancer	CA-125	procedure	*BRCA2* Mutation
3	50	C	2	No	n/a	BSO only	886delGT
5[b]	36	C	1	No	n/a	Hysterectomy/BSO	2041insA
8	52	C	2	No	n/a	Hysterectomy/RSO	7297delCT
10[b]	39	C	5	No	15	Hysterectomy/BSO	2041insA
12	46	AA	2	Yes	4	BSO only	4075delGT
13	52	C	1	Yes	2	Hysterectomy/BSO	5578delAA

[a] CA-125 levels were collected preoperatively. Abbreviations used: C, Causacian; AA, African-American; BSO, bilateral salpingo-oophorectomy.

[b] These patients are sisters.

Table 2

Comparison of the Number of Proteins Identified from Bilateral Collection of Ovarian Surface Epithelium (OSE) and Fallopian Tube Mucosa from Individual Patients[a]

	ovarian surface epithelium proteins			fallopian tube proteins		
patient	**right**	**left**	**both (%)**	**right**	**left**	**both (%)**
3	37	89	22 (17%)	159	138	99 (33%)
5	84	88	56 (33%)	143	129	97 (36%)
8	58	N/A	N/A	N/A	122	N/A
10	80	105	56 (30%)	127	142	84 (31%)
12	64	64	41 (32%)	N/A	136	N/A
13	45	28	17 (23%)	N/A	78	N/A

[a]The number of proteins identified on each side as well as those identified on both sides is given, along with the percentage of proteins that were found bilaterally among those present on either side. Left OSE from patient 8 was unavailable due to prior oophorectomy. Right fallopian tube mucosa was excluded for patients 8 and 13 due to excessive bleeding from the fallopian tube and was unavailable for patient 12 due to scarring of the fimbriated end of the fallopian tube.

Table 3

Subcellular Location of Identified Proteins in Peritoneum, Fallopian Tube, and Ovarian Surface Epithelium

	peritoneum proteins		fallopian tube proteins		ovarian surface epithelium proteins	
cellular compartment	**number**	**percentage**	**number**	**percentage**	**number**	**percentage**
Extracellular	120	18.5%	159	14.6%	120	12.0%
Membrane	135	20.8%	194	17.8%	168	16.7%
Cell surface	13	2.0%	22	2.0%	20	2.0%
Cytoskeleton	40	6.2%	64	5.9%	89	8.9%
Cytoplasm	152	23.4%	319	29.3%	275	27.4%
Intracellular organelles	100	15.4%	174	16.0%	184	18.3%
Nucleus	90	13.8%	156	14.3%	147	14.7%
Total	650	100%	1088	100%	1003	100%

Table 4

Biological Function of Identified Proteins in Peritoneum, Fallopian Tube, and Ovarian Surface Epithelium

	peritoneum proteins		fallopian tube proteins		ovarian surface epithelium proteins	
cellular compartment	**number**	**percentage**	**number**	**percentage**	**number**	**percentage**
Cell communication	77	7.7%	137	8.6%	106	8.1%
Cell death	43	4.3%	66	4.2%	51	3.9%
Cell differentiation	70	7.0%	100	6.3%	85	6.6%
Cell division, growth, and proliferation	43	4.3%	54	3.4%	41	3.1%
Cell organization	77	7.7%	143	9.0%	132	10.0%
Cell motility	22	2.2%	25	1.6%	30	2.3%
Cell homeostasis	17	1.7%	38	2.4%	35	2.7%
Coagulation	22	2.2%	27	1.7%	17	1.3%
Defense response	62	6.2%	76	4.8%	53	4.0%
Development	71	7.1%	113	7.1%	106	8.1%
Metabolic processes	180	17.9%	313	19.7%	254	19.3%
Response to stimulus	121	12.0%	180	11.3%	136	10.3%
Regulation of biological processes	125	12.4%	192	12.1%	156	11.9%
Transport	76	7.6%	126	7.9%	114	8.7%
Total	1006	100%	1590	100%	1316	100%