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Detection of Endometrial Cancer via Molecular Analysis of DNA Collected with Vaginal Tampons

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Abstract

Objective—We demonstrate the feasibility of detecting EC by combining minimally-invasive specimen collection techniques with sensitive molecular testing.

Methods—Prior to hysterectomy for EC or benign indications, women collected vaginal pool samples with intravaginal tampons and underwent endometrial brushing. Specimens underwent pyrosequencing for DNA methylation of genes reported to be hypermethylated in gynecologic cancers and recently identified markers discovered by profiling over 200 ECs. Methylation was

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evaluated individually across CpGs and averaged across genes. Differences between EC and benign endometrium (BE) were assessed using two-sample t-tests and area under the curve (AUC).

Results—Thirty-eight ECs and 28 BEs were included. We evaluated 97 CpGs within 12 genes, including previously reported markers (*RASSF1*, *HSP2A*, *HOXA9*, *CDH13*, *HAAO*, and *GTF2A1*) and those identified in discovery work (*ASCL2*, *HTR1B*, *NPY*, *HS3ST2*, *MME*, *ADCYAP1*, and additional *CDH13* CpG sites). Mean methylation was higher in tampon specimens from EC v. BE for 9 of 12 genes (*ADCYAP1*, *ASCL2*, *CDH13*, *HS3ST2*, *HTR1B*, *MME*, *HAAO*, *HOXA9*, and *RASSF1*) (all $p < 0.05$). Among these genes, relative hypermethylation was observed in EC v. BE across CpGs. Endometrial brush and tampon results were similar. Within tampon specimens, AUC was highest for *HTR1B* (0.82), *RASSF1* (0.75), and *HOXA9* (0.74). This is the first report of *HOXA9* hypermethylation in EC.

Conclusion—DNA hypermethylation in EC tissues can also be identified in vaginal pool DNA collected via intravaginal tampon. Identification of additional EC biomarkers and refined collection methods are needed to develop an early detection tool for EC.

Keywords

endometrial cancer; tampon; Tao brush; methylation; early detection

Introduction

Early detection approaches for endometrial cancer (EC) are lacking, despite the fact that EC is the most common gynecologic malignancy in the United States and in many other developed countries (1, 2). While low-risk, early stage EC has an excellent prognosis with 5-year overall survival (OS) >95%, 5-year OS when diagnosed at stage III or IV is sobering at 68% and 17%, respectively (3). Additionally, while the incidence of EC is disparately higher among Caucasian women compared with African Americans, mortality is higher among the latter (4). As similar care has been shown to eliminate racial disparities in outcomes among women with EC, this survival difference may, in part, reflect barriers to accessing health care and resultant delays in diagnosis (5). Early detection increases the chance of cure (6, 7) and may also allow for minimization of surgical intervention and avoidance of costly adjuvant therapy and its related complications. An early detection method for EC may overcome barriers to care and potentially reduce morbidity and mortality related to this cancer and its treatment.

The prolonged history of irregular bleeding that precedes diagnosis of most ECs (8) offers a window of opportunity to identify at-risk women prior to the development of advanced cancer. Up to 25% of women who develop EC are diagnosed with a precursor lesion, endometrial hyperplasia, between 1 to 20 years prior to their EC diagnosis (9, 10). Additionally, the average progression time to EC from endometrial hyperplasia ranges from >15 years for the mildest degrees of hyperplasia to 4 years for the most severe lesions (11), suggesting there is a lengthy interval during which early detection and intervention is possible. Nonetheless, research on early detection methods is minimal (12-16).

Approximately 20% of women with EC have Papanicolaou tests that contain cells suspicious for EC (17), which demonstrates that spontaneous shedding of tumor cells occurs frequently and is detectable with a relatively insensitive test. Furthermore, Kinde et al, demonstrated that 100% of 24 women with EC could be identified by testing their routinely collected liquid cervical cytology samples for a panel of 12 mutations (16). Prior to that, Fiegl et al demonstrated that DNA from the vaginal pool, collected via tampon, was hypermethylated in 15 women with EC compared to 109 women without EC (18). Taken together, these studies suggest that detection of EC via minimally invasive methods is a promising approach; however, optimization of markers, standardization of collection methods, and improvement in specificity are needed.

Methylated DNA released from necrotic tumor cells is an attractive target and has been detected in a variety of biological fluids, including sputum, serum, peritoneal fluid, stool, nipple aspirates, urine, and vaginal fluid (18-20). DNA methylation assays offer potentially higher sensitivity than cytology as 1) intact cells are not required and 2) the relative stability of DNA offers advantages compared with protein or RNA based tests. A growing number of genes have been identified as methylated in EC and its precursors. *RASSF1* is methylated in both primary and recurrent EC, as well as in morphologically normal appearing endometrium adjacent to ECs, irrespective of menopausal status (18, 21-23); *MLH1* methylation occurs in atypical hyperplasia as well as EC suggesting that it is an early event in EC carcinogenesis (22, 24-28). *CDH13*, *PR-B*, *ERalpha-C*, *CIDEA*, *HAAO*, *RFXP3*, *CDKN2A*, *PTEN*, *p16*, *APC*, *HSPA2*, *SOCS2*, and *MGMT* are also hypermethylated in EC (18, 22, 29-33) and additional candidate methylation markers are emerging (18, 27, 33-35).

Pairing innovative, low cost, and minimally-invasive biospecimen sampling techniques with sensitive molecular testing that can be analyzed efficiently using high through-put methods has already led to the development of a novel screening and early detection tool for colorectal cancer (20, 36). Minimally-invasive biospecimen collection techniques to sample the endometrium are currently available (37-39). However, a more convenient mode of biospecimen collection that employs a well-accepted hygiene product, the intravaginal tampon, would offer the advantage of patient acceptance and the ability to self-collect specimens; such a tool could be particularly useful for women with barriers to health care access. Towards that end, we assessed methylation of both established and recently identified candidate genes as biomarkers for EC within the vaginal pool, collected via intravaginal tampon, from women with and without EC, to assess the feasibility of such an approach as an early detection method for EC.

Experimental Design

Subject Eligibility Criteria

Women undergoing clinically-indicated hysterectomy were prospectively enrolled at the Mayo Clinic, Rochester, Minnesota from April 24, 2009 through January 25, 2011. Women eligible for this pilot study included those with a new diagnosis of complex atypical hyperplasia or any histology of biopsy-proven EC. The benign endometrium (BE) control group included women 45 years of age undergoing hysterectomy for benign indications. Women were excluded if they had recurrent cancer, known cervical cancer, received

neoadjuvant chemotherapy or radiotherapy, prior tubal ligation, endometrial ablation, cervical stenosis, active endometriosis on treatment, or prolapse precluding tampon retention within the vagina. Patient, tumor, and biospecimen collection data were abstracted. This study was approved by the Mayo Clinic and National Cancer Institute Institutional Review Boards. Women were excluded from this analysis if both the tampon and Tao brush were not successfully collected.

Biospecimen collection

The biospecimens collected for this study included the vaginal pool (collected via intravaginal tampon), endometrial brushings (collected via Tao brush) (37), and primary tumor tissue. Patients were instructed to refrain from intercourse and douching for 24 hours prior to surgery. On the day of their hysterectomy, women self-placed an intravaginal tampon that was to be retained within the vagina for at least 30 minutes prior to surgery. After induction of anesthesia, the tampon was removed and placed in phosphate buffered saline (PBS). Subsequently, an endometrial brushing with a Tao brush was performed and the brush tip was removed and suspended in PreservCyt (Hologic; Bedford, MA). Duration of vaginal tampon retention and time to DNA extraction for tampon and Tao brush were recorded.

Biomarker selection

Thirteen candidate biomarkers for methylation analysis were selected from two sources for this study. Genes selected from the gynecologic cancer literature were chosen based on their documented hypermethylation in gynecologic cancer (*RASSF1*, *HSP2A*, *HOXA9*, *CDH13*, *HAAO*, and *GTF2A1* (18, 21-23, 29, 40-42)). Additional genes were identified via methylation profiling utilizing the Illumina GoldenGate Standard Cancer Methylation Panel, which includes 1,505 CpG sites from 807 genes, in 143 ECs from the Polish Endometrial Cancer Study (PECS) study, 69 ECs from the Endometrial Hyperplasia Study (EH), and 63 BE samples from the Benign Reproductive Tissue Evaluation Study (BRTE) study. Details on the discovery effort to identify these abnormally methylated genes and the affected CpG sites have been previously reported (34). We ultimately selected CpG sites from the eight top candidates from the discovery effort (*ASCL2*, *HTR1B*, *NPY*, *HS3ST2*, *MME*, *ADCYAP1*, *SOX1*, *CDH13*) for further evaluation. All sites had probes that had p values $<10^{-7}$ in comparisons of methylation levels in EC versus BE, methylation differences between BE and EC >0.4 , and mean methylation values in BE <0.2 (34).

DNA extraction from tampon and Tao brush

DNA was extracted from tampon supernatant using the Roche High Pure Viral Nucleic Acid kit per manufacturer's protocols after spinning the tampon in a 50mL conical tube atop a removable sterile stainless steel sieve. DNA was eluted in 50 μ L of elution buffer and concentration and purity were measured from absorbance at 230 nm, 260 nm, 280 nm on a Nanodrop-2000 spectrophotometer.

Prior to DNA extraction from each Tao brush sample, the brush was scraped into the PreservCyt vial to remove remaining cells and material from the brush and discarded. After centrifugation, DNA was extracted from the pellet using the Puregene Tissue Core Kit A

(Qiagen) according to the manufacturer's protocols. DNA was eluted in 80 μ L of elution buffer and concentration and purity were measured from absorbance at 230 nm, 260 nm, 280 nm on a Nanodrop-2000 spectrophotometer.

Bisulfite modification

DNA underwent bisulfite modification using the EZ-96 DNA Methylation Gold kit (Zymo Research, Irvine, CA) according to manufacturer's protocols. One μ g of DNA was used as input per sample and bisulfite-modified DNA was eluted in 30 μ L elution buffer for pyrosequencing.

Methylation analyses

Pyrosequencing was performed separately on tampon and Tao brush DNA. Primers were designed using Pyrosequencing Assay Design Software (Qiagen) (Supplementary Table 1) and the CpG coordinates are listed in Supplementary Table 2. Pyrosequencing assays were developed for *RASSF1A*, *HSP2A*, *HOXA9*, *CDH13*, *HAAO*, and *GTF2A1* utilizing previously published primers (18) or designed based on CG rich sites within the promoter for genes without previously published primers. These assays covered between 4 and 16 CpG sites. Pyrosequencing assays were also developed for *ASCL2*, *HTR1B*, *NPY*, *HS3ST2*, *MME*, *ADCYAP1*, *SOX1*, and additional *CDH13* sites covering between 2 and 10 CpG sites overlapping with the probes included in the Illumina Golden Gate array (34). Pyrosequencing assays for *SOX1* failed to perform adequately.

A target region of up to 250 bp was amplified by PCR using paired primers complementary to the bisulfite-treated DNA sequence for each site and both methylated and unmethylated sequences of selected genes were amplified. Amplification was carried out on 20 ng of bisulfite treated DNA using TaqGold DNA polymerase (Applied Biosystems) under the following conditions: 10 min at 95°C, followed by 50 cycles of 35 sec at 95°C, 35 sec at 57.5°C, and 1 min at 72°C. The resultant PCR products were checked by gel-electrophoresis to confirm the size of the product and rule out the possibility of primer dimers. Pyrosequencing reactions were performed on a Biotage PyroMark MD System (Qiagen) according to manufacturer's protocols. The incorporated biotinylated primer was immobilized on streptavidin-coated beads to purify and render the denatured, single stranded and biotinylated PCR product. The single-stranded product was then annealed to 0.3 μ M of the sequencing primer complementary to the single-stranded template, and placed at 85 °C for 2 min, cooled to room temperature for 5 min. The pyrosequencing reaction was then performed by the sequential addition of single nucleotides in a predefined order. Raw data were analyzed using the provided Pyro Q-CpG 1.0.9 analysis software (Qiagen).

Statistical methods

Correlation of methylation levels for CpG sites within a gene, as well as association of methylation across genes, were assessed using Spearman correlation coefficients, presented graphically as pairwise comparisons across sites (Figure 1). In addition, principal components were utilized to assess the total variation among CpG sites within a gene (43). The number of principal components needed to explain 90% of the total variation in the

gene was reported along with the percent of the gene's variation captured by those principal components.

Patient and tumor characteristics were tabulated and compared for women with EC and BE by Wilcoxon rank sum and Fisher's exact tests. The primary analysis was the association of gene-level methylation between EC and BE samples using the mean of the percent methylation for all CpG sites within a gene. Methylation differences between EC and BE were assessed using two-sample t-tests and receiver operating characteristics area under the curves (AUC).

Results

Patient and tumor characteristics

Specimens from 38 patients with biopsy-proven EC and 37 BE controls were prospectively collected. Complete biospecimens (both tampon and Tao brush) were collected from all 38 EC and 28 BE subjects and were included in the subsequent analyses. Patient and tumor characteristics are listed in Table 1. Among cancer cases, the 2009 FIGO stage distribution was as follows: 26 (68.4%) IA, 6 (15.8%) IB, 3 (7.9%) IIIC, and 2 (5.3%) stage IVB. There was also one case of complex atypical hyperplasia (CAH) which was included in the EC group. Histology distribution was as follows: 1 (2.6%) CAH, 31 (81.6%) endometrioid, 3 (7.9%) serous, and 3 (7.9%) clear cell. The EC group was older and had higher median BMI than women with BE; the rate of hormone replacement use was the same in the two groups (Table 1).

Uterine pathology among the BE group included: adenomyosis (n=1; 3.6%); benign endometrial polyp (n=1; 3.6%); leiomyomas alone (n=5; 17.9%) or associated with hyperplasia without atypia (n=1; 3.6%), tubo-ovarian abscess (n=1; 3.6%), benign ovarian mass (n=7; 25%), or benign ovarian mass and endometriosis (n=2; 7.1%). Final diagnoses for the remainder of the BE group included menorrhagia/dysmenorrhea without structural abnormality (n=2; 7.1%) and benign ovarian mass alone with no uterine pathology (n=5; 17.9%). One incidental endocervical adenocarcinoma in situ and 2 unexpected leiomyosarcomas were retained in the control group.

DNA yields for Tao brush and tampon samples

The median amount of DNA collected from the vaginal pool via the tampon was 4.7 μ g [IQR 2.5, 10.7 μ g] and from the endometrial Tao brush 33 μ g [IQR 8.1, 62.6 μ g]; both collection techniques yielded sufficient DNA quantities for large-scale methylation analyses via pyrosequencing. Agarose gel electrophoresis and PCR of GAPDH confirmed high quality DNA from both tampon and Tao brush biospecimens.

Factors associated with methylation

We evaluated methylation within the tampon biospecimens according to patient and tumor characteristics as listed in Table 1. The strongest associations between gene-level methylation and patient or clinical characteristics were correlation of higher *ASCL2* and *RASSF1* methylation with greater tumor diameter ($R^2=0.24$, $p=0.005$, and $R^2=0.16$,

$p=0.016$, respectively) and lower *NPY* methylation in patients with advanced stage disease (IIIC/IV) (mean $5.6 \pm 2.6\%$) compared to patients with early stage disease (CAH, IA/IB) (mean $19.9 \pm 18.2\%$, $p=0.044$). Age, BMI, cancer grade, cancer histology, myometrial invasion, and lymphovascular space invasion were not significantly associated with methylation differences at the gene level. Similar results were found for the Tao brush samples.

Median (IQR) time between diagnostic endometrial sampling and tampon biospecimen collection within the EC cohort was 15 (12, 20) days. The duration of time between diagnosis and vaginal pool sampling was not significantly related to methylation percentage. Additionally, the duration of tampon retention within the vagina did not influence methylation percentage (all $R^2 < 0.08$) or DNA yield (all $R^2 < 0.08$). Median (IQR) tampon retention time for the EC group was 111 (90.5, 163.5) minutes and 98 (90.3, 115) minutes for the BE group ($p=0.91$). Methylation results across all 12 genes among tampons collected from the 3 women with unexpected neoplasias (2 leiomyosarcomas and 1 endocervical adenocarcinoma in situ) were similar to those from women with a benign final pathology diagnosis.

Differential DNA methylation in tampon samples discriminates EC from benign endometrium

Among the 12 genes, 97 CpG sites were evaluated via pyrosequencing, ranging from 2-16 sites for each gene. Site-specific methylation differences between EC and BE are listed in Supplementary Table 3. There was strong correlation in methylation levels across CpG sites within a gene, with the exception of *GTF2A1* (Figure 1, Tables 2 and 3). Of note, none of the 10 CpG sites within *GTF2A1* exhibited significant differences in methylation between EC and BE within tampon or Tao brush specimens (Supplementary Table 3).

In gene-level analyses, significantly higher methylation ($p < 0.05$) was observed in tampon specimens from EC compared to BE for 9 of the 12 genes studied (*ADCYAP1*, *ASCL2*, *CDH13*, *HS3ST2*, *HTR1B*, *MME*, *HAAO*, *HOXA9*, and *RASSF1*) (Table 2). The genes with the greatest differences in mean methylation between EC and BE were *HTR1B* (23.8 ± 14.9 vs. 10.4 ± 4.2 , $p < 0.0001$, AUC=0.82), *HOXA9* (21.5 ± 21.8 vs. 6.0 ± 4.8 , $p = 0.0001$, AUC=0.74), and *RASSF1* (21.3 ± 18.1 vs. 6.8 ± 5.3 , $p = 0.0001$, AUC=0.75) (Figure 2). *HTR1B*, *HOXA9*, and *RASSF1* had similar patterns of methylation in EC and BE Tao brush samples, with slightly higher absolute methylation percentages in Tao brush samples compared to the tampon samples (Figure 2). While 6 genes identified previously via profiling (*ADCYAP1*, *ASCL2*, *CDH13*, *HS3ST2*, *MME*, and *NPY*) demonstrated comparably high levels of association (AUCs ranging from 0.66-0.70) between methylation and EC status, *HTR1B* had the highest AUC of all genes tested at 0.82 (Figure 3a). Among the genes reported in the literature, *HAAO*, *HOXA9*, and *RASSF1* had AUCs comparable to those identified in our biomarker discovery work, ranging from 0.68-0.75, while *HSP2A* and *GTF2A1* showed no association between methylation and disease status (Figure 3b).

Analyses of Tao brush DNA demonstrated higher levels of methylation compared to tampon analyses (Figure 2, Supplementary Table 3); however differences in methylation levels between EC and BE were similar in the vaginal tampon and Tao brush samples (Figure 2).

Among genes identified by profiling, *ADCYAP1* had the greatest increase in methylation percentage among EC (37.9 ± 20.0) compared to BE (11.6 ± 6.5) with an excellent AUC of 0.90; among genes previously reported, *RASSF1* performed the best with mean methylation percentage of 26.0 ± 14.7 among EC vs. 10.5 ± 4.6 among BE (AUC=0.82) (Table 3).

Discussion

This proof of principle study demonstrates that EC-specific DNA methylation markers are detectable in lower genital tract samples collected via intravaginal tampon. Our results are supported by prior studies showing detection of EC markers using DNA methylation to test tampons (18) and mutation analysis of liquid-based cervical cytology samples (16). Specifically, our study shows that women with EC have higher gene-level methylation of both well-studied and recently identified markers in both vaginal tampon samples as well as endometrial brushings compared to women with BE.

A collection method that is easily performed with a familiar sampler, such as a tampon, can potentially enable self-collection and delivery to a testing laboratory by mail. This approach could extend access to EC early detection methods into settings with limited resources secondary to socioeconomic status, geography, or cultural barriers. Among women who are traveling to distant referral centers for consultations, obtaining assay results in advance of visits could enable appropriate triage to gynecologic oncology or general gynecology clinics. In addition, the potential to use serial collection of tampons in research and clinical management may have value, especially among women at increased risk for EC secondary to high penetrance germline mutations, a history of endometrial hyperplasia, or treatment with tamoxifen. Given the rising incidence of EC in the United States and elsewhere, disparities in health care access, and the high mortality associated with metastatic disease (7, 44, 45), the development of an early detection approach has the potential to streamline management of at-risk women. In addition, recognizing that metrorrhagia and postmenopausal bleeding are common and disturbing symptoms, the ability to rule out EC can provide reassurances and limit the need for repeated biopsies.

Previous studies have successfully exploited minimally-invasive collection techniques and highly sensitive DNA methylation analyses to detect cancer at other organ sites. DNA methylation changes that occur in colorectal cancer (20, 36) and upper gastrointestinal (GI) cancer (46, 47) can be detected in stool. In fact, Imperiale, et al recently demonstrated that multi-target stool DNA testing is a highly sensitive screening test for colorectal cancer that carries a detection rate similar to that of colonoscopy (36). This novel test has the potential to substantially impact colon cancer screening and supports momentum to develop novel and minimally-invasive screening tests for other cancers.

In this study, we validated methylation markers previously identified as hypermethylated in EC compared with BE (18, 21-23, 29, 34, 40), and demonstrated detection of these markers in the downstream effluent of the female reproductive tract. One of these markers, *HS3ST2*, was identified in our original discovery efforts (34) and has recently been validated as an EC biomarker in a cohort of women with endometrial biopsies showing atypical hyperplasia (48). In addition, we identified a novel gene methylated in EC, *HOXA9*, which is also highly

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sites was consistent within each candidate gene that discriminated between EC and BE; single CpG sites per gene could therefore be utilized to simplify the DNA analyses. At present, additional discovery efforts using more global methylation analyses are underway to identify other novel candidate biomarkers that may better separate EC from BE. In addition, DNA mutations detected in the lower female genital tract have also been shown to discriminate between EC and BE (16), somatic mutations in genes, such as *TP53*, *PIK3CA*, and *PTEN*, are well described in EC (40, 50-52), and further discovery utilizing next generation sequencing is underway in our sample set. As such, DNA methylation and mutation analyses could complement one another in the detection of EC.

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A major strength of this study is the inclusion of genes that are well-documented as methylated in EC (18, 21-23, 29, 40) as well as more recently defined, highly methylated genes validated in independent sets of EC and BE tissues (34). DNA biomarkers are attractive for designing a self-collected test because it may enable at-home collection and mailing to a testing laboratory. In addition, DNA aberrations can be assessed with high throughput methods allowing rapid assessment of multiple samples simultaneously, the cost of methylation and mutation analyses are progressively decreasing, and the increasing number of certified laboratories that can perform molecular analysis with rigorous quality assurance would support the clinical use of self-collected tampons, once a standardized approach is validated.

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A notable limitation of this study is that all ECs had biopsy-proven disease via pre-study endometrial sampling. As such, while the median time between diagnostic endometrial sampling and vaginal pool collection was greater than two weeks and methylation percentages were not associated with the length of time between sampling and tampon collection, the impact of biopsy on tumor cell and cell-free DNA shedding is unknown. Spontaneous cancer cell shedding has been demonstrated to occur in EC (17) and all subjects with EC in our study presented with abnormal vaginal bleeding, which suggests ongoing spontaneous tumor DNA shedding. Nonetheless, interpretation of our study and previous reports are limited by collection of samples following prior endometrial instrumentation (16, 18). Further investigation of the degree of DNA methylation occurring within the vaginal pool of prebiopsied symptomatic and high-risk asymptomatic women is warranted to further assess spontaneous DNA shedding from EC. Additionally, the impact of endometrial sampling on vaginal pool DNA methylation is warranted and prospective biospecimen collections in these patient groups are currently underway. Ultimately, determination of whether vaginal pool methylation changes can be identified in the general female population to identify those without risk factors for EC but at risk for EC as well as a cost-analysis of screening the general population are warranted.

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In summary, this proof of principle study demonstrates that self-collection of the vaginal pool via intravaginal tampon in combination with sensitive molecular analyses may enable detection of EC. Future directions include extension of DNA methylation marker analyses to additional novel genes, inclusion of EC mutations that could complement and improve EC detection sensitivity, and analyses of DNA collected via tampons prior to endometrial biopsy among high risk women. This approach may yield an early detection test for EC,

which could enable effective treatment with reduced morbidity and improve mortality from the disease, particularly among under-served women.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- Genes hypermethylated in primary endometrial cancer tumors can be detected in endometrial brushing and tampon biospecimens
- Lower genital tract biospecimen collection via tampon was well-accepted by women in this study
- Combining methylation analyses and a minimally-invasive biospecimen collection could yield a novel screening test for endometrial cancer

Figure 1.

Graphical representation of Spearman correlation coefficients across genes and CpGs. Methylation among CpGs within each gene is highly correlated, with the exception of *GTF2A1*. *GTF2A1* was included as a negative gynecologic cancer control as it is hypermethylated in ovarian cancer but has not been shown to be hypermethylated in endometrial cancer.

Figure 2.

Jit plots of absolute methylation percentages as determined in tampon and Tao biospecimens from women with endometrial cancer (case) and benign endometrium (control) for (a) *HTR1B*, (b) *HOXA9*, and (c) *RASSF1* show consistently higher methylation within biospecimens collected from women with endometrial cancer. Further discovery efforts are ongoing to identify additional methylated genes in EC and other molecular markers that further separate EC from BE.

Figure 3.

Receiver operating characteristic (ROC) curve analyses with areas under the curve (AUC) are shown for discrimination between endometrial cancer and benign endometrium using methylation markers. (a) Six novel candidate genes identified via prior profiling and *CDH13* (additional CpGs identified on prior profiling) and (b) five genes identified from the gynecologic cancer literature. *HAAO* and *RASSF1* have been published as hypermethylated in endometrial cancer. *HSP2A* and *GTF2A1* have not been shown to be hypermethylated in endometrial cancer, but are methylated in ovarian cancer. *HOXA9* hypermethylation in endometrial cancer is a novel finding in this study. Colored lines show ROC curves for individual genes.

Table 1

Clinical characteristics among women with endometrial cancer and benign endometrium.

	Endometrial cancer (N=38)	Benign endometrium (N=28)	P value
Age, years (median, IQR)	60 (56.8, 70)	55 (49, 67.5)	0.034*
BMI, kg/m ² (median, IQR)	33.1 (27, 38.3)	26.8 (22.7, 32.8)	0.0049*
Prior HRT use (n, % yes)	9 (25%)	6 (21%)	0.78 [#]
Stage, 2009 FIGO (n, %)		---	---
CAH	1 (2.6)		
IA	26 (68.4)		
IB	6 (15.8)		
IIIC	3 (7.9)		
IVB	2 (5.3)		
Grade (n, %)		---	---
CAH	1 (2.6)		
1	21 (55.3)		
2	8 (21.1)		
3	8 (21.1)		
Histology (n, %)		---	---
CAH	1 (2.6)		
Endometrioid	31 (81.6)		
Serous	3 (7.9)		
Clear cell	3 (7.9)		
Maximum primary tumor diameter, cm (median, IQR)	3.4 (2.8, 4.8)	---	---
Myometrial invasion, % (median, IQR)	20.0 (5.0, 41.0)	---	---
Lymphovascular space invasion (n, % yes)	6 (15.8%)	---	---

* Wilcoxon rank sum test

[#] Fisher's exact test

Performance of methylation markers in tampon samples obtained from women with endometrial cancer and from women with benign endometrium.

Table 2

Gene	Benign endometrium (N=28)					Endometrial cancers (N=38)				
	CpG positions (N)	PC (N)	Total variation (%)	Mean methylation (%)	Std Dev	Mean methylation (%)	Std Dev	P-value	c-stat	
Discovery										
<i>ADCYAPI</i>	4	1	97.6	18.1	11.6	28.4	18.3	0.0078	0.665	
<i>ASCL2</i>	5	3	94.6	10.9	10.0	22.4	20.5	0.0097	0.675	
<i>CDH13</i>	13	2	91.4	18.2	15.4	28.1	18.1	0.0392	0.670	
<i>HS3ST2</i>	10	4	90.7	12.6	7.4	21.4	17.8	0.0190	0.672	
<i>HTR1B</i>	2	2	100.0	10.4	4.2	23.8	14.9	<0.0001	0.820	
<i>MME</i>	3	2	93.2	11.0	7.7	20.5	14.9	0.0033	0.702	
<i>NPY</i>	2	1	98.2	6.6	13.2	17.9	17.5	0.2855	0.658	
Literature										
<i>GTF2AI</i>	10	7	92.5	3.9	1.6	3.4	1.1	0.1812	0.570	
<i>HAAO</i>	10	1	96.5	10.4	15.0	21.9	20.3	0.0240	0.679	
<i>HOXA9</i>	14	2	92.7	6.0	4.8	21.5	21.8	0.0001	0.741	
<i>HSP2A</i>	4	2	96.8	29.2	26.5	29.9	18.3	0.9124	0.527	
<i>RASSF1</i>	16	2	92.3	6.8	5.3	21.3	18.1	0.0001	0.753	

Performance of methylation markers in Tao brush samples obtained from women with endometrial cancer and from women with benign endometrium.

Table 3

Benign Endometrium (N=28)									Endometrial cancers (N=38)			
Gene	CpG positions (N)	PC (N)	Total Variation (%)	Mean Methylation (%)	Std Dev	Mean Methylation (%)	Std Dev	P-value	e-stat			
Discovery												
ADCYAP1	4	1	97.8	11.6	6.5	37.9	20.0	<0.0001	0.901			
ASCL2	5	3	95.8	7.3	8.9	18.9	23.5	<0.0001	0.818			
CDH13	13	3	93.1	21.5	8.9	36.9	19.0	0.0001	0.752			
HS3ST2	10	3	92.7	11.5	5.2	25.8	15.9	<0.0001	0.799			
HTR1B	2	2	100.0	17.5	16.3	25.1	16.8	0.0880	0.682			
MME	3	1	94.5	10.6	4.4	28.0	19.3	<0.0001	0.856			
NPY	2	1	96.9	19.2	14.0	23.9	17.8	0.2800	0.579			
Literature												
GTF2A1	10	7	92.0	2.7	0.3	3.0	0.8	0.0100	0.672			
HAAO	10	1	96.5	9.3	9.2	13.5	22.9	0.0004	0.644			
HOXA9	14	2	84.8	20.5	9.5	29.6	18.7	0.0138	0.618			
HSP2A	4	2	94.5	25.9	13.9	32.4	19.0	0.1100	0.596			
RASSF1	16	3	92.5	10.5	4.6	26.0	14.7	<0.0001	0.820			