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Insights into the field carcinogenesis of ovarian cancer based on the nanocytology of endocervical and endometrial epithelial cells

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Ovarian cancer ranks fifth in cancer fatalities among American women. Although curable at early stages with surgery, most women are diagnosed with symptoms of late-stage metastatic disease. Moreover, none of the current diagnostic techniques are clinically recommended for at-risk women as they preferentially target low-grade tumors (which do not affect longevity) and fail to capture early signatures of more lethal serous tumors which originate in the fimbrae region of the fallopian tubes. Hence, the early detection of ovarian cancer is challenging given the current strategy. Recently, our group has developed a novel optical imaging technique, partial wave spectroscopic (PWS) microscopy, that can quantify the nanoscale macromolecular density fluctuations within biological cells via a biomarker, disorder strength (L_d). Using the concept of field carcinogenesis, we propose a method of detecting ovarian cancer by PWS assessment of endometrial and endocervical columnar cells. The study includes 26 patients (controls = 15, cancer = 11) for endometrium and 23 (controls = 13, cancer = 10) for endocervix. Our results highlight a significant increase in L_d (% fold-increase > 50%, p-value < 0.05) for columnar epithelial cells obtained from cancer patients compared to controls for both endocervix and endometrium. Overall, the quantification of field carcinogenic events in the endometrium and the novel observation of its extension to the cervix are unique findings in the understanding of ovarian field carcinogenesis. We further show independent validation of the presence of cervical field carcinogenesis with micro-RNA expression data.

Key words: ovarian cancer, field-carcinogenesis, endocervix, nano-architecture, endometrium

Additional Supporting Information may be found in the online version of this article.

Significance: This work has threefold significance for ovarian cancer: ***Biologically***, we detect nano-architectural alterations in the histologically "normal"-appearing cells of the endometrium and endocervix for ovarian field carcinogenesis. We validate the novel observation of cervical origin using miRNA expression-levels. ***Clinically***, these results have the potential to translate into a minimally invasive early detection technique. ***Technologically,*** we demonstrate that PWS nanocytology is exquisitely sensitive to cell nano-architecture at length scales < 300nm, a paradigm shift in biomedical optics.

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The development of cancer is a complex and cumulative multistep process in which discrete genetic and epigenetic events (*e.g.*, profound activation of proto-oncogenes, loss of tumor-suppressor genes, promoter hypermethylation, histone acetylation, *etc.*) transform microscopically normal-appearing mucosa to premalignant tissue to frankly malignant tissue and ultimately to metastasis. Several recent reports have established that apparently "normal" cells (*i.e.*, away from the actual tumor location) undergo similar genetic/epigenetic molecular changes as the actual cancer cells, giving rise to the phenomenon of field carcinogenesis.[1] Field carcinogenesis (or field-effect/field-defect/field-cancerization) is the concept that the genetic/environmental milieu that results in a neoplastic lesion in one area of the organ is diffusely present either in the organ itself or in neighboring organs that share the same "field of injury."[1] Hence, field carcinogenesis provides a fertile mutational milieu throughout the organ with stochastic events leading to focal neoplastic lesions.[2] Examples of this phenomenon include colorectal, lung, pancreatic, esophageal, breast, ovarian, cancers[1,3] indicating that it is universal throughout different organs. For example, in colon cancer numerous biomarkers obtained from the visually normal rectal mucosa are correlated with proximal neoplasia including cellular (apoptosis[4] and proliferation[5]), molecular (genomic[6] and proteomic[7]), micro-architectural[8] and morphological.[9] This effect has also been observed in adjacent organs in various disease states, termed as "extended" field carcinogenesis.[1]

What's new?

Ovarian cancer is frequently fatal because it is not detected until it has progressed to late-stage metastatic disease, and new techniques for early detection are sorely needed. Sometimes, apparently normal cells can undergo changes similar to the cancer cells, in a phenomenon called "field carcinogenesis." In these instances, multiple cancers arise in different sites within the same region. In this paper, the authors employ a novel technique, partial wave spectroscopy (PWS) to detect ovarian field carcinogenesis by looking for nanoscale changes in the architecture of endometrial and cervical cells. This report signifies the first demonstration that PWS nanocytology can detect cellular structures at such a small scale. Using this technique to detect ovarian field carcinogenesis could represent a minimally invasive approach to early intervention for ovarian cancer.

For example, the buccal (cheek) epithelium is considered a "molecular mirror" for lung carcinogenesis[10] while duodenal mucosa has been identified as a surrogate site for pancreatic cancer.[1,11] Similarly, uterine lining (*i.e.*, endometrium) has been identified as a surrogate site for ovarian field carcinogenesis.[1] Herein, we further investigate the field effect in ovarian cancer.

Ovarian cancer ranks fifth in cancer-related mortality in American women. There are approximately 21,990 new cases and 15,460 deaths reported in 2011.[12] The 5-year survival rate is approximately 90% when the cancer is confined only to the ovary (stage I), while it drops to 33% when the disease is diagnosed at stage III or IV.[13] Unfortunately, most women (more than two-thirds) are diagnosed with late-stage metastatic disease (*i.e.*, in stage III or IV) because early (surgically curable) disease is clinically silent. The current screening options (*e.g.*, serum marker CA-125, HE4; transvaginal doppler-ultrasound/ultrasonography) are suboptimal for detecting more lethal and commonly found (60–80% malignancies) serous tumors.[13,14] Hence, identifying early stage high-grade serous tumors to study premalignant or early lesions poses an uphill challenge.

In this scenario, the possible application of the field carcinogenesis approach for ovarian cancer is underscored by the fact that the serous subtype originates in the distal fimbrae of the fallopian tubes[14,15] while other areas of the gynecological tract (*e.g.*, endometrium and endocervix) are contiguous with the fallopian tubes. Specifically, the endometrium has been implicated as a surrogate site for ovarian field carcinogenesis[1] due to the presence of frequent synchronous endometrial tumors in ovarian cancer patients[1,16] and specific gene methylation in the microscopically normal endometrial mucosa.[17] Herrinton *et al.*[18] have suggested that the incidence of synchronous primary endometrial and ovarian cancers are twofold to 10-fold higher than the incidence of each cancer alone, and that both have several common risk factors and hence could occur together in the same women with relatively high frequency. In addition, Russell *et al.* have demonstrated the multifocal origin of tumorigenesis in the upper female genital tract wherein most of the serous ovarian cancers had multiple primary neoplasia extended to the endometrium in spite of being confined to the ovaries.[19] Moreover, the three main subtypes of ovarian tumors (serous, endometrioid and mucinous) are morphologically identical to carcinomas of the fallopian tubes, endometrium, and endocervix, respectively. However, the important question is to sensitively and efficiently quantify the field carcinogenic events from the endometrium and the endocervix.

Recently, our group has developed a novel optical imaging technique, partial wave spectroscopic (PWS) microscopy, which has proved its potential in detecting field carcinogenic signatures in different organs such as colon, lung, pancreas and esophagus.[20–24] PWS provides sub-diffractional sensitivity to length scales of ~10–20 nm.[21,25] Furthermore, we have previously reported that the PWS-measured biomarker, disorder strength (L_d), measures nanoscale consequences of subtle genetic/epigenetic perturbations in colon carcinogenesis using colon cancer cell lines (*i.e.*, genetically altered but microscopically similar-looking human adenocarcinoma cell lines, HT29) and animal models (*i.e.*, intestinal neoplasia MIN mouse and azoxy-methane treated AOM rat model).[25] In both these models, L_d was parallel to the aggressiveness or neoplastic potential of the cells. In essence, L_d increase is a hallmark of field carcinogenesis in different organs and a marker of increased neoplastic potential in microscopically indistinguishable but genetically altered cells. Herein, we attempt to determine whether we could sensitively detect the ovarian field carcinogenic alterations in normal-appearing endometrium and endocervix using PWS nanocytology.

In this manuscript, we present a proof-of-concept study using PWS nanocytology performed on columnar epithelial cells of the endometrium and endocervix of women having benign ovarian disease (*e.g.*, cystadenoma, fibroma) and those having frank ovarian carcinomas. Our results from both endometrium and endocervix show a gradient increase in the nano-architectural biomarker, disorder strength (L_d), from control to cancer patients based on field carcinogenesis. Thus, for the first time we quantify field carcinogenic alterations in the endometrium. Moreover, we report a novel observation of the presence of field carcinogenesis in the cervix. We further validate cervix origin for field carcinogenesis with microRNA (miRNA) expression data. miRNAs are small, noncoding, single-stranded RNAs which have emerged as important regulators of gene expression.[26] Their role in early carcinogenesis is implicated due to the fact that approximately 50% of miRNAs are located in fragile areas (deletion/

amplifications). Specifically, in ovarian carcinogenesis, miRNA modulation has been demonstrated (*i.e.*, most miRNAs are downregulated[27]) and some of these miRNAs represent ideal targets for detection, diagnosis and/or therapy.[28] Thus, we report the unique quantification of field carcinogenic signatures in the endometrium with the novel finding of these changes in the cervix, and display an opportunity for early ovarian cancer detection using PWS nanocytology as a minimally invasive technique.

Material and Methods

Clinical sample preparation

All studies were performed and samples were collected with the approval of the Institutional Review Board at Northshore University HealthSystem. Patients undergoing counseling for hysterectomy were approached for consent prior to surgery. After the surgical specimen was removed and taken to the pathology gross room, a pathologist used a cytology brush on the endometrium and cervix separately. Using these cytology brushes, cell-smears from both endometrium and cervix were prepared, then fixed in 95% ethanol and finally subjected to PWS analysis by an investigator blinded to patient diagnosis. Although the cytology slide contained different types of cells including epithelial, inflammatory, red blood cells, and so forth, we note that all the measurements reported here were taken from only the columnar epithelial cells for both the endometrium and cervix (although the endocervix has a population of squamous epithelium). There was no cross-contamination during the surgical procedure. This was made possible by staining each patient slide with standardized hematoxylin and cytostain staining protocol and directly visualizing the cells before taking the PWS measurements (the PWS system contains a flipper mirror that directs the image of a cell onto a digital camera for quick visualization).

miRNA profiling

Total RNA was isolated from the cervical mucosal brushings (smears) using Ribopure RNA kit (Ambion) as per the manufacturer's instructions. We performed miRNA profiling of the cervical mucosa from ovarian cancer patients ($n = 4$) and matched controls ($n = 4$) using Taqman low density miRNA array cards (Applied Biosystems, Foster City, CA). Total RNA from the cervical cells was reverse transcribed by priming with a mixture of stem-looped primers (MegaPlex RT Primers Human Pool A, Applied Biosystems) using the Taqman miRNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. The miRNA profiling was done using Taqman Array Human miRNA Panel A (v.3, Applied Biosystems). The cDNA diluted in Universal PCR Master Mix II (Applied Biosystems) was then loaded on to the Taqman Low Density Array microfluidic cards and run on the ABI 7900 HT real time PCR system using manufacturer's instructions. Relative concentrations of miRNAs were calculated using the comparative ($2^{-\Delta\Delta Ct}$) method and MammU6 was used as the endogenous control for data normalization. Real time PCR data analysis was done using the RQ Manager 1.2.1 (Applied Biosystems) and RealTime StatMiner software (Integromics, Philadelphia, PA).

PWS microscopy system

The in-depth explanation of the PWS instrument used for this study and the theory behind the technique are reported in Supporting Information as well as in the references.[25,29] In brief, a spatially incoherent white light illuminates a specimen and the reflected backscattered image is projected on to a CCD camera (Princeton Instruments, NJ) through a liquid crystal tunable filter (LCTF; CRi, Woburn, MA; spectral resolution = 7 nm). The spectral fluctuations of the backscattered light (from 500 to 700 nm) are further analyzed for every pixel of the obtained image (1 pixel = 6.45 μm). In essence, the interference of multiple reflections from macromolecular complexes within a cell with the reflection from the top surface results in the PWS signal. The underlying physical phenomenon of PWS (based on mesoscopic light transport theory[30]) is that the optical interference of backscattered light waves is sensitive to the spatial variations of optical refractive index at any subdiffractional length scale (limited to signal-to-noise). Because refractive index is a linear function of local macromolecular mass density (DNA, RNA, proteins, *etc.*),[31] PWS quantifies these nanoscale refractive-index fluctuations (and thus, spatial fluctuations in macromolecular density) in a parameter called disorder strength (L_d). Theoretically, L_d is defined as: $L_d = \delta n^{\alpha} \times l_c^{\beta}$ where δn is the standard deviation of the refractive index (*i.e.*, mass density) variations, and l_c is the correlation length of these variations. In a cell, δn is defined by the inhomogeneity of macromolecular density and l_c is the characteristic size of the intracellular structures. The coefficient α depends on the refractive index contrast of the medium on top of a cell relative to its internal fluctuations (*i.e.*, the cytology sample preparation) and is equal to 1 in our case, while β depends on the configuration of the optical setup (*i.e.*, collection numerical aperture of the system) and is $\sim$1 for the instrumentation used in this study. As the optical refractive index is linearly related to the local mass density of macromolecules (that is, refractive index $n = n_o + \kappa\rho$ where n_o is the refractive index of the medium, ρ is the portion of intracellular solids by volume which is mass density, and κ is the specific refractive index increment with a constant value of $\sim$0.186 ml/g for most of the intracellular compounds[29,31]), $L_d \propto \delta n \propto \kappa\delta\rho$. Thus, L_d is a measure of the spatial variations of macromolecular density and increases with macromolecular condensation.[32] The exact nature of the compaction depends on the specific intracellular location where L_d is increased. For example, if L_d increases at a particular location in the nucleus, this may correspond to chromatin condensation at that specific location.[23,33] The value κ can be increased (thus increasing the local refractive index for a given macromolecular concentration) by certain cytological stains such as hematoxylin and eosin, leading to the

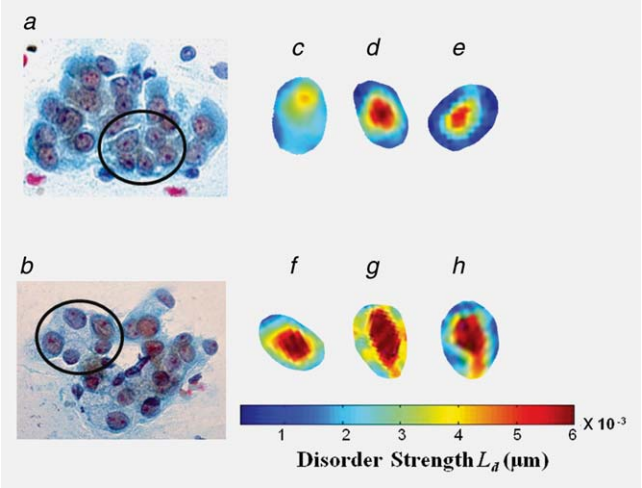

Figure 1. Representative microscope and PWS-generated images: (*a*) and (*b*) are the traditional microscope images (in the transmission mode) of the normal-appearing and stained (hematoxylin and cytostain) endometrial columnar epithelium from control and cancer patient respectively. (*c*–*e*) are the representative PWS-generated pseudo-color heat-maps of the circled region from control patient while (*f*–*h*) are for the cancer patient. It is evident that although the columnar epithelial cells of control and cancer patient look microscopically identical, the disorder strength (L_d) was clearly augmented for cells from cancer patient compared to the control.

enhancement of the signals originating from intracellular structures[34] and therefore increasing the sensitivity of PWS nanocytology to alterations in cell nano-architecture of the stained structures. Hence, staining can alter the nanoscale disorder and in this case, it enhances the L_d-differences between control and cancer cells.

Essentially, PWS readout is an image [two-dimensional (2D) map] of a cell showing the intracellular distribution of disorder strength $L_d(x,y)$. Using this 2D map, the mean intracellular disorder strength (the average over x and y pixels) is obtained. The average for a group of cells (~50–70 cells for each patient) is calculated and defined as the mean disorder strength per patient, $L_d^{(P)}$. The cumulative average is then calculated over all the patients of a specific group and is termed the group mean of the disorder strength, $L_d^{(g)}$ while its standard deviation is defined as $\sigma^{(g)}$. This average disorder strength $L_d^{(g)}$ and the standard error calculated from its standard deviation $\sigma^{(g)}$ are depicted in all the bar plots in this report. Hence, the standard error bars are calculated over the total number of patients for each category, representing the standard error of the mean, $L_d^{(g)}$.

Statistical methods

First, we determined the number of cells to be measured for each slide (to balance between accuracy and practicality) and calculated the total number of cells adequate for PWS analysis. Using power analysis, we observed that $n = 15$ cells would yield 95% power to detect a between-group difference for a two-sided t-test at a 0.05 significance level. However, we used $n > 30$ cells for each patient. Therefore, we decided to target this number for each patient in this study. To accurately measure the performance of L_d, we calculated the statistical parameters: % difference of the mean L_d and p-values on the data. Mean-differences (denoted by %Difference) provided the % fold-increase between two populations. The p-values were calculated using standardized Student's t-test on the total number of patients in control and cancer group for both the endometrium and endocervix. A two-tailed p-value (assuming unequal variances) of 0.05 or less was considered to be statistically significant in this study. All the parameters were calculated using Microsoft Excel (Microsoft Corporation, Redmond, WA). Statistical Software, STATA (StataCorp LP, College Station, Texas) was used to generate analysis-of-covariance (ANCOVA) p-values and AUROC (area-under-the receiver operator characteristic curve) test statistics.

Results

Detection of field carcinogenesis in endometrium

For each cell, PWS microscopy generates a 2D image of disorder strength, $L_d(x,y)$, that is, spatial distribution of L_d within a cell. Figures 1*a* and 1*b* show representative traditional light microscope images (in the transmission mode) of stained columnar endometrial cells obtained from a control and a cancer patient, respectively. Although the images appear microscopically indistinguishable (and hence suggest no obvious alterations at microscopic length scales >400 nm), the pseudo-color heat maps of localized distribution of L_d indicate nanoscale perturbations (Figs. 1*c*–1*e* *vs.* 1*f*–1*h*). The red color-coded regions represent higher L_d in the cells from cancer patients compared to control cells, implying the nanoscale sensitivity of PWS.

Our first task was to measure field carcinogenic changes in the endometrium using PWS nanocytology. To do so, we examined the microscopically normal-appearing columnar epithelial cells obtained from the endometrium of $n = 26$ patients who underwent a hysterectomy procedure. There were $n = 15$ patients with benign disease (forming the control group) while the remaining $n = 11$ patients had frank cancers (*i.e.*, all the subtypes, I–IV). After performing PWS nanocytology on these patients, we observed a gradient increase in disorder strength (denoted as ΔL_d) in the endometrial epithelial cells obtained from cancer patients compared to the controls as depicted in Figure 2*a*. This ΔL_d was statistically significant (%Difference = 53, p-value = 0.01) as highlighted in Table 1. This result is unique as it presents the first quantification of the nano-architectural alterations in the endometrial mucosa which is contiguous with the fallopian tubes. Our results are consonant with the observation that endometrial mucosa may be altered in ovarian field carcinogenesis.[1] There are a few recent studies which strengthen our findings: (i) the presence of concurrent endometrial tumors is a common observation in ovarian cancer.[16,35] Moreover, approximately 10% of women diagnosed with ovarian cancer have synchronous endometrial cancer and ~5% of women initially diagnosed with

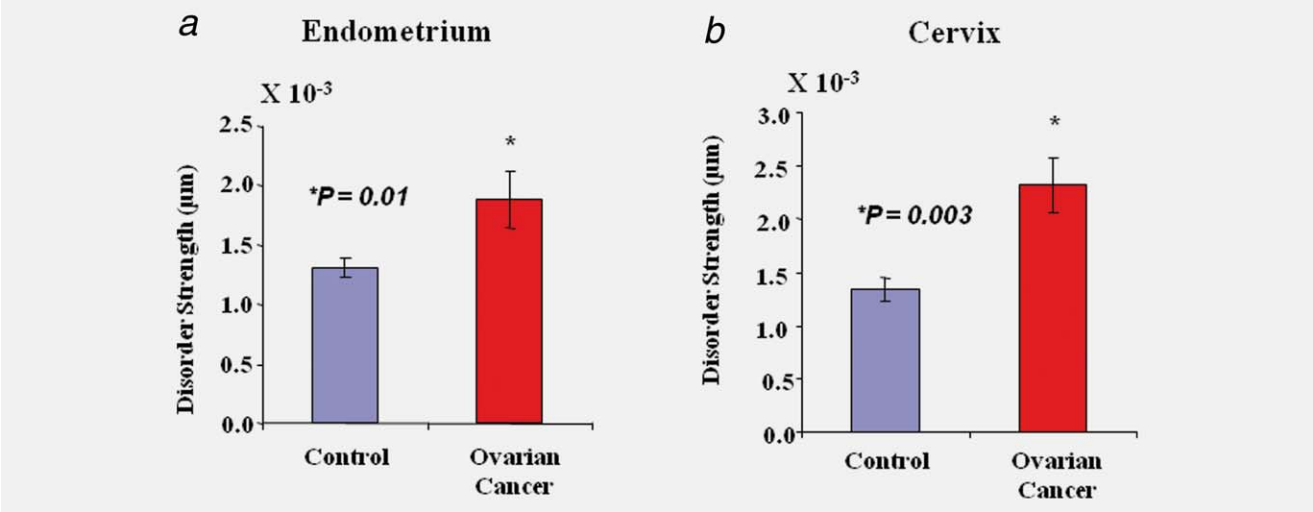

Figure 2. PWS results for control and cancer patients from endometrium and endocervix: (*a*) demonstrates the overall results obtained from control ($n = 15$) and cancer ($n = 11$) patients based on PWS-analysis performed on normal-appearing endometrial columnar epithelium. It depicts that the L_d is significantly higher (%fold-increase = 53, $p = 0.01$) for cancer patients compared to controls implying the sensitivity of L_d to field carcinogenesis in endometrium. Similarly, (*b*) represents the results for L_d obtained from the normal-looking columnar epithelium of endocervix from control ($n = 13$) and cancer ($n = 10$) patients. There appears to be significant (%fold-increase = 73, $P = 0.003$) field carcinogenic alterations in endocervix for cancer patients compared to controls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

endometrial cancer will eventually develop ovarian cancer. Importantly, these synchronous tumors are second primaries rather than metastatic disease.[35] (ii) Widschwendter *et al.*[17] evaluated DNA methylation of the polycomb group target, HOXA, which is a common event in carcinogenesis. They noted that HOXA9 independently portended a 12.3-fold increased risk of ovarian cancer and 14.8-fold increased risk for early stage disease independent of age, phase of the menstrual cycle and histology of the cancer.[17] (iii) Ovarian and endometrial cancers share many of the same risk factors, and (iv) chemopreventive agents such as progestins seem to work on both cancer types.[36] However, we would like to note that patients with endometrial cancer were rejected from our study in order to avoid any confounding. Overall, PWS nanocytology results provide the first proof of quantification of field cancerization in the endometrial cells.

Table 1. The statistical performance of the PWS analysis for endometrium and endocervix

Statistical parameters	Cervix	Endometrium
Number of patients	13 *vs.* 10	15 *vs.* 11
% Difference (fold-increase)	73%	53%
p-value	0.003	0.01

This table highlights number of patients used for each organ, %mean-differences (%fold-increase) and the *p*-values. All the statistical parameters are calculated for the PWS-measured parameter, disorder strength (L_d).

Presence of field carcinogenesis in Cervix

Although we obtained promising results from the endometrium, it is still a relatively invasive site in terms of clinical investigation. Hence, we asked the question whether field carcinogenic changes would be present in the most easily accessible part of the gynecological tract, the cervix, as it is contiguous with the endometrium and the fallopian tubes. To answer this, we employed the same strategy of obtaining cytological smears from the endocervical region of the patients who underwent a hysterectomy. We obtained measurements from the columnar epithelium of $n = 13$ benign disease controls and $n = 10$ ovarian cancer patients. As demonstrated in Figure 2*b* and Table 1, using PWS nanocytology, we acquired a statistically significant ΔL_d (%Difference = 73, p-value = 0.003) for cancer patients compared to the controls. Although we have a modest dataset, the performance of a single biomarker, L_d is remarkable in terms of identifying these nanoscale ultrastructural changes in microscopically normal-looking cervical epithelium. This, as per our knowledge, is the first evidence for the presence of extended field cancerization in the endocervix for ovarian cancer patients and hence opens a new avenue for further investigation using other well-established techniques.

miRNA expression data confirming the field carcinogenesis in Cervix

Although there are reports confirming the possibility of field cancerization for the endometrium,[1,16] there are none implicating the cervical field directly. However, we wanted to explore the molecular mechanisms responsible for these field carcinogenic changes. We next wanted to verify the PWS results for cervical field carcinogenesis using another well-established and independent technique that provides

nanoscale sensitivity. To this end, we performed miRNA analysis on the cervical smears of a few patients to understand the biological mechanism underlying these architectural changes. We used the cervical brushing from $n = 4$ control and $n = 4$ cancer patients and obtained miRNA expression data from them as described in the Methods section. As depicted in Figure 3, we noted that several of the miRNAs including miR187, miR199, miR211, miR424, miR432, miR495, miR674, and so forth, were significantly downregulated (% Difference $\geq$ -70%, $P < 0.005$) in the cancer patients as compared to controls. Interestingly, a few of these miRNAs, namely miR187, miR199, miR211, miR424 and miR432 were found to be significantly downregulated even in serous ovarian carcinomas and epithelial ovarian cancers.[26,27,37] Thus, miRNA expression data strengthened the PWS nanocytology-based results and provided independent validation of the endocervix as a potential surrogate site for extended field carcinogenesis in ovarian cancer.

Diagnostic performance of endometrial and endocervical disorder strength

As mentioned earlier, PWS nanocytology was sensitive to field carcinogenic alterations in both endocervix and endometrium. Hence, we next wanted to evaluate the diagnostic performance of this approach in a clinical setting. We do understand that we have a modest dataset, however as a proof-of-principle we obtained an excellent area under receiver operator characteristic (AUROC) curve of 85% for the cervix and 81% for endometrium. Furthermore, L_d from the cervix had sensitivity of 80% with a specificity of 88% while the endometrial L_d had sensitivity of 80% and specificity of 78%. In comparison to other available screening options (*e.g.*, CA-125 has sensitivity of 60%[38]), PWS nanocytology, combined with the field carcinogenic concept, provides improved and promising performance characteristics for detecting early ovarian cancer signatures, *albeit* on a limited dataset.

We next wanted to assess whether diagnostic performance would improve by combining PWS-measured L_d from both endometrium and endocervix. First, we checked how the L_d values and its diagnostic performance compared for patients with cytology slides obtained from both the surrogate organs. We had $n = 8$ controls and $n = 8$ cancer patients for whom the cytological smears were obtained from both sites. As highlighted in Table 2, the performance was better for the endometrium compared to the cervix (AUROC = 88% *vs.* 80%). This could happen due to the proximity of the endometrium to the ovaries, which are the primary tumor location. The correlation coefficient between cervical and endometrial L_d values was 0.23, suggesting a weak, *albeit* significant, correlation ($p = 0.04$) between the two. Phenomenologically, a positive correlation between endocervical and endometrial L_d is not surprising as both sites are part of the extended ovarian field carcinogenesis. Upon combining the L_d values from the endocervix and the endometrium, we observed a significant improvement in the diagnostic performance as represented in Table 2. The AUROC improved to 97%, which is substantial compared to the performance of each individual organ.

Finally, we investigated the effect of demographic risk factors on the disorder strength values. Specifically, age has been implicated as one of the well-characterized risk factors for ovarian carcinoma with a predicted higher risk for women above age 50.[39] Hence, we performed ANCOVA and noted no significant confounding with age ($p = 0.75$ for L_d) in both organs as indicated in Table 3. The other common risk factors such as drinking and smoking, are not well correlated with risk of ovarian cancer, and L_d was similarly found to be independent of them ($p = 0.16$ for smoking and 0.36 for drinking). Thus, the nano-architectural biomarker L_d is not confounded by demographic risk factors.

Discussion

Herein, we show that PWS analysis of normal-appearing columnar epithelial cells from both endometrium and endocervix can serve as a minimally invasive, easy-to-perform, sensitive and efficient technique for detecting early field carcinogenic events in ovarian cancer. Our results based on women having benign disease and frank ovarian carcinoma suggest that the PWS-measured biomarker, disorder strength (L_d), is significantly higher for cancer patients compared to controls in both these organs. This proof-of-concept study is the first to report the extension of field cancerization to the cervix and is unique in terms of quantifying field carcinogenic changes in the endometrium. Furthermore, miRNA expression data independently confirms the finding of cervical field carcinogenesis. We also demonstrate the diagnostic performance of PWS based on the single biomarker L_d for both cervix and endometrium on the modest dataset presented in this study. Our results indicate that L_d is independent of confounding parameters such as age, drinking and smoking.

The primary issue with ovarian cancer is the detection of the lethal serous subtype at an early stage. Most of the current screening techniques fail to capture these lesions. The most important reason for this poor performance is the etiology of ovarian cancer where the more aggressive serous subtype does not follow the traditional adenoma-carcinoma sequence.[15] Specifically, ovarian cancers can be dichotomized into: (i) high-grade serous tumors (leading cause of ovarian cancer mortality) and (ii) low-grade mucinous tumors (that do not impact the longevity and are the targets of CA-125, transvaginal ultrasound). The low grade lesions are believed to originate on the surface epithelium of the ovary while the serous subtype initially develops in the fimbrae of the fallopian tubes (*i.e.*, outside the ovary) and both have different genetic mutational make-up.[15] The data reviewed by Levanon *et al.*[15] indicated the secretory epithelial cells of the fallopian tubes (FTSECs) as the origin of serous ovarian tumors. This was confirmed by Salvador *et al.*[40] where they evaluated serous malignancies and noted that the copy number of

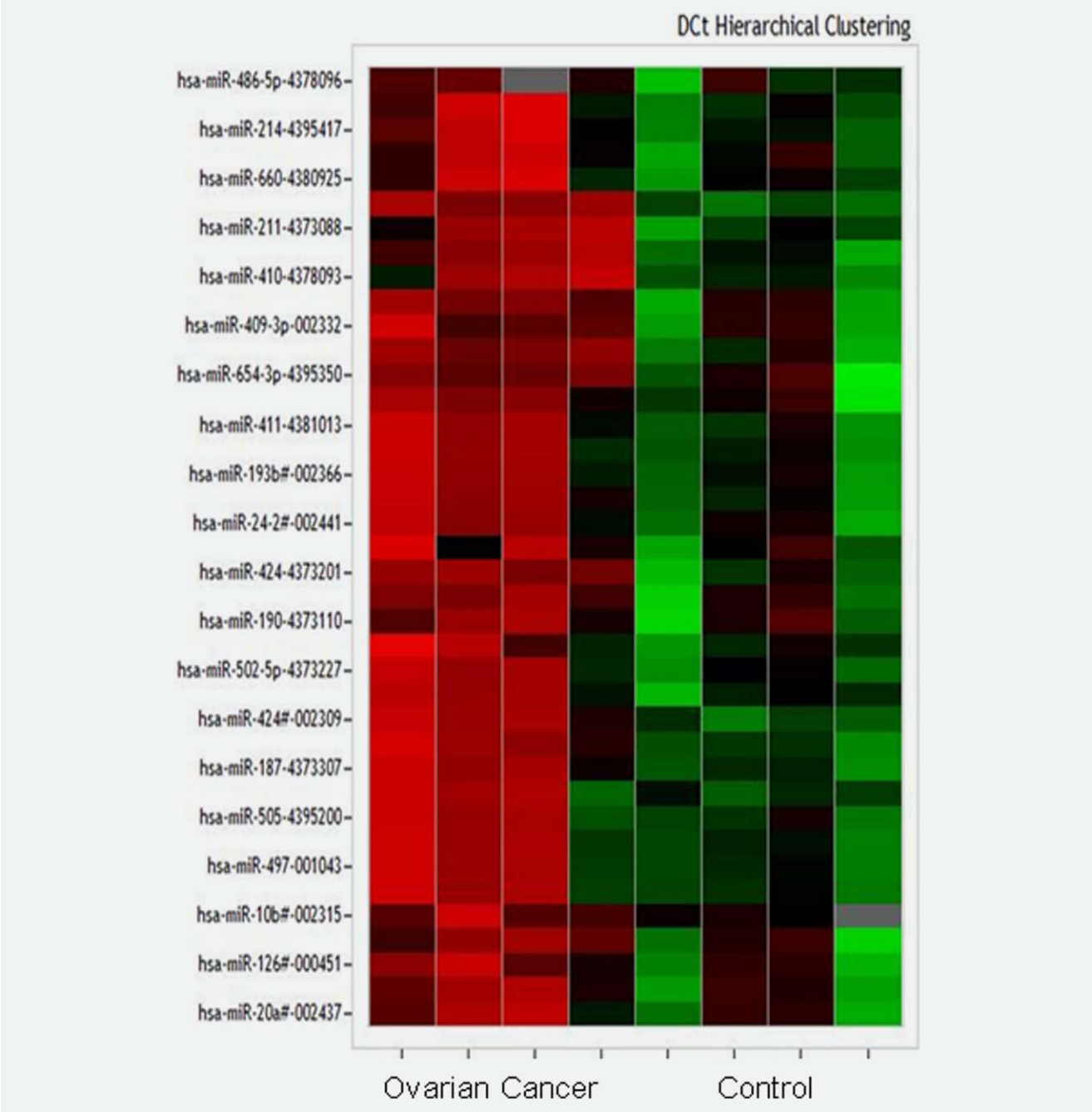

Figure 3. demonstrates the micro-RNA (miRNA) expression data obtained from the endocervical smears of control ($n = 4$) and cancer ($n = 4$) patients. The data suggest significant downregulation of some of the key miRNAs in cancer patients that have been implicated for ovarian cancer.

abnormalities detected in the tumor (irrespective of whether the bulk of the tumor was in the ovary, peritoneum or fallopian tube) had the same detectable genetic alterations as the microscopically normal fallopian tubes, suggesting a monoclonal origin. Moreover, tubal ligation has been shown to decrease ovarian cancer risk by ~50%,[41] further supporting the origin of serous tumors. Hence, the challenge is to identify serous lesions at an early stage in a minimally invasive way.

Several lines of evidence suggest that there are early genetic/epigenetic and consequently morphological changes that give rise to field carcinogenic changes in various cancers such as colon, lung, pancreas,[1,6,7] and it has also been reported in organs farther away from the primary tumor location, termed "extended" field carcinogenesis.[1,10] This concept can be applied to ovarian cancer as the serous tumors originate in the fallopian tubes and the other areas of the gynecological tract (*e.g.*, endometrium and endocervix) are

Table 2. Compares the performance of PWS-analysis for endometrium and endocervix

Patients with slides for both organs	Average L_d of control	Average L_d of cancer	%Difference	*p*-value	AUROC	Sensitivity	Specificity
Cervix ($n = 8$ *vs.* 8)	1.41×10^{-3}	2.24×10^{-3}	60%	0.03	80%	75%	88%
Endometrium ($n = 8$ *vs.* 8)	1.19×10^{-3}	2.00×10^{-3}	67%	0.02	88%	75%	100%
Both organs Combined ($n = 16$ *vs.* 16)	1.30×10^{-3}	2.12×10^{-3}	63%	0.001	97%	100%	87%

This table compares the mean-values, %fold-increase and *p*-values for L_d obtained from columnar epithelium of control ($n = 8$) and cancer ($n = 8$) patients for whom cytology smears were obtained from both endocervix and endometrium. Furthermore, it highlights the diagnostic performance such as area under the receiver-operator curve (AUROC), sensitivity and specificity for PWS-based analysis for endometrium and endocervix separately and also when both the organs are combined. The results imply that the diagnostic performance improves significantly when both endocervical and endometrial L_d are combined.

Table 3. The impact of demographic factors on the single biomarker, L_d

Demographic Factors	Cervix control	Cervix cancer	Endometrium control	Endometrium cancer	Effect on L_d,ANCOVA *p*-value
Age (mean $\pm$ SD) years	59 ± 12	61 ± 11	57 ± 12	62 ± 11	0.75
Smoking (%Smokers)	46	20	38	20	0.16
Drinking (%Alcoholic)	7	10	15	20	0.36

This table shows that L_d is not confounded by demographic risk-factors such as age ($p = 0.75$), smoking ($p = 0.16$) and drinking ($p = 0.36$).

contiguous with the fallopian tubes. Specifically, the endometrium has been identified as a surrogate site for ovarian field carcinogenesis.[1] These findings open a possible avenue for assessing field carcinogenic alterations in endometrial mucosa which is contiguous with the fallopian tubes. Our results using PWS nanocytology measured these ultrastructural changes in endometrial columnar epithelium with good performance (AUROC of 81%) despite a limited dataset. Thus, PWS results provide the very first quantification of field cancerous changes in the endometrium.

Although the endometrium (or uterine lining) provides a promising site for field cancerization, it is still quite invasive clinically. This led us to investigate the most easily accessible site, the cervix, which is contiguous with the endometrium and fallopian tubes and hence can be a target for field carcinogenic signatures. The endocervix has not yet been implicated as a surrogate site in the literature, however, our results indicated a promising performance (AUROC = 85%) in terms of detecting field effect changes. We further validated these results using miRNA expression data obtained from the cervical smears of the same patients. Specifically, we observed significantly lower expression of some of the miRNAs (*e.g.*, miR187, miR199, miR211, miR432 and miR424) that were found downregulated even in serous ovarian carcinoma and epithelial ovarian cancer.[26,27] Our observation that field cancerization could be extended to the cervix is significant as it opens up a novel aspect of ovarian field carcinogenesis which requires further explanation and understanding.

An obvious question that needs to be addressed is the confounding effect of human papilloma virus (HPV) in ovarian cancer due to the presence of HPV genome (*e.g.*, HPV 16, HPV 18 DNA) in some cases of serous and mucinous tumors.[42] However, it has been noted that patients with cervical cancer actually have a lower risk of developing either synchronous or late second primary tumors in the ovaries (specifically in the nonradiation therapy group).[42–45] We note that none of the patients in this study had HPV infection and hence the PWS-observed effect in cervix should be primarily due to field carcinogenesis, and could provide an unbiased indicator of potential risk of ovarian cancer. Recently, Kinde *et al.*[46] identified specific and similar somatic mutations in the DNA collected during liquid pap-smear in HPV negative patients suffering from endometrial and ovarian cancers. This report although supports our work, it hypothesizes the origin of these mutations to tumor metastasis.

Even though disorder strength (L_d) appears to be a potential biomarker for quantifying field carcinogenesis compared to conventional approaches, the biological rationale needs further explanation. In PWS analysis, L_d represents spatial variations in the local macromolecular mass density of cellular building blocks (proteins, RNA, DNA, ribosome, cytoskeleton, *etc.*) while an increase in L_d corresponds to macromolecular condensation (*e.g.*, chromatin looping in the nucleus leads to higher local mass density, hence higher refractive index variation and correlation length resulting in higher L_d). Our recent work suggests that these changes occur both in cytoplasm and the nucleus. We have recently reported that pharmacological disruption of specific components of the cytoskeleton in both the nucleus and cytoplasm normalized ΔL_d between genetically altered colon cancer cell lines.[29] Furthermore, L_d increase in the nucleus indicates chromatin condensation, which was substantiated by our group using numerical simulations,[47] electron microscopy[48] and histone deacetylase 2 (HDAC2) overexpression[33] in early

colon carcinogenesis. In particular, for ovarian cancer the miRNA data highlights some of the first-order events which would lead to higher order effects resulting in cancer progression. For example, miRNAs govern the response of the ovarian cancer cells to chemotherapy as reported by Boren *et al.*[49] Hence, L_d could measure the nanoscale morphological consequences of all these epigenetic modulators.

There are limitations with our approach that need to be acknowledged. First, we report an excellent proof-of-principle performance of the PWS marker, L_d in both endometrium and endocervix, *albeit* on a modest sample size. We realize that to validate our approach, we need to conduct studies over a large prospective population. Second, ovarian cancer is a heterogeneous disease with several distinct histologies (*e.g.*, serous, mucinous, adenocarcinoma, clear cell carcinoma, stromal, germ-cell, *etc.*) that are not part of current study. Future studies will attempt to include all these various subtypes of patients. Third, we report performance based on a single biomarker, L_d; however, identifying more PWS-derived markers can improve the performance characteristics. Fourth, although we are the first group to report the presence of extended field carcinogenesis in the cervix using miRNA as a supporting proof, it needs to be further understood by other techniques. An obvious extension would be to study the gene targets of some of the key miRNAs, specifically in cervix samples from normal and cancer patients.

A few recent reports suggest that combining more serum markers (*e.g.*, combining serum markers CA-125 and HE4) or employing two separate modalities help in reducing unnecessary surgeries and strengthening early detection.[50] We envision that PWS nanocytology could be applied in combination with other available screening techniques (*e.g.*, CA-125 serum markers or ultrasound) to improve the early diagnosis of ovarian cancer.

In conclusion, we provide the first observation that interrogation of the endocervical and endometrial columnar epithelium with PWS can quantify nano-architectural alterations in ovarian field carcinogenesis. Although these results are obtained from a small dataset, we believe that if confirmed on a larger prospective population PWS could improve the early detection of ovarian cancer. Overall, this report opens a new dimension in ovarian cancer screening program and adds a novel facet in the understanding of ovarian field carcinogenesis by implicating cervical mucosa.

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