Nanoscale markers of esophageal field carcinogenesis: potential implications for esophageal cancer screening

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Background and study aims: Esophageal adenocarcinoma (EAC) has a dismal prognosis unless treated early or prevented at the precursor stage of Barrett's esophagus-associated dysplasia. However, some patients with cancer or dysplastic Barrett's esophagus (DBE) may not be captured by current screening and surveillance programs. Additional screening techniques are needed to determine who would benefit from endoscopic screening or surveillance. Partial wave spectroscopy (PWS) microscopy (also known as nanocytology) measures the disorder strength (L_d), a statistic that characterizes the spatial distribution of the intracellular mass at the nanoscale level and thus provides insights into the cell nanoscale architecture beyond that which is revealed by conventional microscopy. The aim of the present study was to compare the disorder strength measured by PWS in normal squamous epithelium in the proximal esophagus to determine whether nanoscale architectural differences are detectable in the field area of EAC and Barrett's esophagus.

Methods: During endoscopy, proximal esophageal squamous cells were obtained by brushings and were fixed in alcohol and stained with standard hematoxylin and Cyto-Stain. The disorder strength of these sampled squamous cells was determined by PWS.

Results: A total of 75 patient samples were analyzed, 15 of which were pathologically confirmed as EAC, 13 were DBE, and 15 were non-dysplastic Barrett's esophagus; 32 of the patients, most of whom had reflux symptoms, acted as controls. The mean disorder strength per patient in cytologically normal squamous cells in the proximal esophagus of patients with EAC was 1.79-times higher than that of controls ($P<0.01$). Patients with DBE also had a disorder strength 1.63-times higher than controls ($P<0.01$).

Conclusion: Intracellular nanoarchitectural changes were found in the proximal squamous epithelium in patients harboring distal EAC and DBE using PWS. Advances in this technology and the biological phenomenon of the field effect of carcinogenesis revealed in this study may lead to a useful tool in non-invasive screening practices in DBE and EAC.

Introduction

A revolution has occurred in the management of Barrett's esophagus with the incorporation of endoscopic therapies to treat patients with high-grade dysplasia (HGD) and even early esophageal adenocarcinoma (EAC) [1]. However, there remain significant questions concerning management. Current screening practices are ineffective in capturing all patients at risk of Barrett's esophagus and have yet to be proven to be cost effective [2,3]. It is often quoted that the rate of progression from non-dysplastic Barrett's esophagus (NDBE) to EAC is 0.5% per year. More recent studies suggest that estimated incidence conveys a much lower progression of 0.12%–0.27% per year [4,5]. In addition, endoscopic surveillance has not been proven to be cost effective [6]. Given the low but present risk, screening and surveillance strategies may be optimized to focus on those patients at risk of progression. A relatively non-invasive tool to screen patients for esophageal cancer to identify those patients who are not being captured by a Barrett's esophagus screening and surveillance program is needed.

Advanced optical techniques capable of differentiating between dysplasia and metaplasia in Barrett's esophagus are being developed [7–10]. However, the analysis of differences in the optical properties between known dysplasia and non-dysplastic tissue will need to provide risk stratifi-

cation beyond traditional histology in order to provide a clinical impact.

Meanwhile, several studies using gene analysis and metabolic profiling have established alterations that occur in the normal lining of esophageal epithelium surrounding the tumor or dysplastic lesion as a result of the field effect of carcinogenesis [11, 12]. In one comprehensive study of the genetic profile of the normal squamous epithelium in patients with EAC, Barrett's esophagus, and controls, a carcinogenic field effect was detected in 52 % of the genes analyzed, including *Bax, BFT, CDX2, COX2, DAPK, DNMT1, GSTP1, RARα, RARγ, RXRα, RXRβ, SPARC, TSPAN*, and *VEGF* [11].

The field effect, introduced by Slaughter in 1957 [13], is the proposition that the genetic/environmental milieu that results in a neoplastic lesion in a particular tissue site should also be detectable outside this location [14, 15]. These field effect changes have been noted in a range of cancers, suggesting processes in the field area that resemble the initial changes towards carcinogenesis that may provide a fertile ground for subsequent neoplastic changes. In the esophagus, reliable detection of these field effect alterations could potentially be used for the risk stratification of patients with Barrett's esophagus without the need to identify and sample the specific area of neoplasia.

An ideal technology to take advantage of the field effect is biophotonics. Recently, partial wave spectroscopy (PWS) microscopy – also known as nanocytology – has demonstrated sensitivity to nanostructural alterations in the histologically normal-appearing mucosa in the field area of colon, lung, and pancreatic cancers that may include remodeling of higher-order chromatin structures and cytoskeletal organization [16 – 18]. PWS performs quantitative imaging of the intracellular disorder strength (L_d – a measure of the spatial heterogeneity of mass distribution of macromolecules), thus quantifying the nanoscale architecture of a cell and its compartments (e. g. the nucleus). Researchers may capitalize on the ability of PWS to detect these nanoscale changes in the tumorigenic field to provide further risk stratification beyond that which is demonstrated by conventional histology via targeted biopsies. Furthermore, PWS analysis of the proximal esophagus may be done using samples collected with or without an endoscopic procedure.

The aim of this study was to assess the disorder strength (L_d), as measured by PWS, in normal squamous epithelium in the proximal esophagus to determine whether nanoscale architectural differences are detectable in the field of EAC and Barrett's esophagus.

Methods

Sampling of the proximal esophagus

Two medical centers – the University of Chicago Medical Center, Chicago, Illinois and Northshore University Health System, Evanston, Illinois – participated in the study. Patients were enrolled to the study between October 2010 and June 2012. The study was approved by the institutional review board of each center. Informed consent was obtained from each patient.

Patients were divided into four groups: 1) patients with EAC, including those with intramucosal carcinoma, tumor limited to the mucosal lining; 2) dysplastic Barrett's esophagus (DBE), patients with Barrett's esophagus and either HGD or low-grade dysplasia (LGD); 3) patients with NDBE; and 4) controls. The control group was defined as those patients who did not have Barrett's esophagus or EAC but who had existing or a history of reflux symptoms or acid suppressive medications.

Inclusion criteria for EAC, DBE, and NDBE groups included patients undergoing esophagogastroduodenoscopy (EGD) for endoscopic surveillance or treatment of Barrett's esophagus or staging of EAC with EGD and endoscopic ultrasound. Exclusion criteria included the inability to provide consent, neoplasia elsewhere in the upper gastrointestinal tract, and prior endoscopic ablative therapy on the esophageal mucosa.

Patients who presented for a screening endoscopy for Barrett's esophagus were categorized as controls if there was no evidence of Barrett's esophagus or placed in the appropriate group based on endoscopic and histological evidence from the procedure. If the procedure during which the samples were taken demonstrated a higher pathology than the original indication for screening endoscopy, the patient was re-categorized based on the higher pathology. A physician who reviewed both the findings on the endoscopy and histology reports as well as previous pathology reports, if applicable, confirmed group placement for each patient.

Procedure and tissue acquisition

During the endoscopic procedure, proximal esophageal squamous cells were obtained using a standard endoscopic cytology brush (Cytology Brush; Cook Medical, Winston Salem, North Carolina, USA) at 15 – 20 cm from the incisors. The brush was then smeared onto glass slides. Specimens were fixed with alcohol and stained with standard hematoxylin and Cyto-Stain (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Sampling of the buccal mucosa

Patients who presented to the endoscopy procedure unit as described above or to the thoracic surgery clinic at the University of Chicago Medical Center and Northshore University Health System participated in the study. Patients were divided into two groups: those patients with EAC, including those with intramucosal carcinoma (tumor limited to the mucosal lining); and patients who presented for screening colonoscopy in the procedure unit without a history of cancer or Barrett's esophagus.

Partial wave spectroscopic microscopy

PWS microscopy is a biophotonics technique that measures the nanoscale distribution of mass density. The instrument and theory of PWS are described in detail in Subramanian et al. [16]. In brief, spatially incoherent white light is incident on a biological cell and its magnified reflection image is detected by a charge-coupled device camera. The spectral fluctuations of the backscattered light (from 500 – 700 nm) are analyzed for every pixel of the obtained image. In essence, the PWS technique breaks up the 3-dimensional intracellular volume into a number of 1-dimensional cylindrical channels; the multiple interference of backscattered light waves from each of those channels results in the PWS signal.

The underlying physical phenomenon utilized by this technique is that the optical interference of backscattered light waves is sensitive to the spatial variations in the optical refractive index at subdiffractional length scales. As a result, whereas the lateral resolution of PWS is diffraction limited, in the axial direction PWS is sensitive to all sizes (limited only by the signal-to-noise ratio of the system). In addition, for essentially all biologically relevant macromolecules (DNA, RNA, proteins, lipids, etc.), the refractive index is a linear function of the local macromolecular

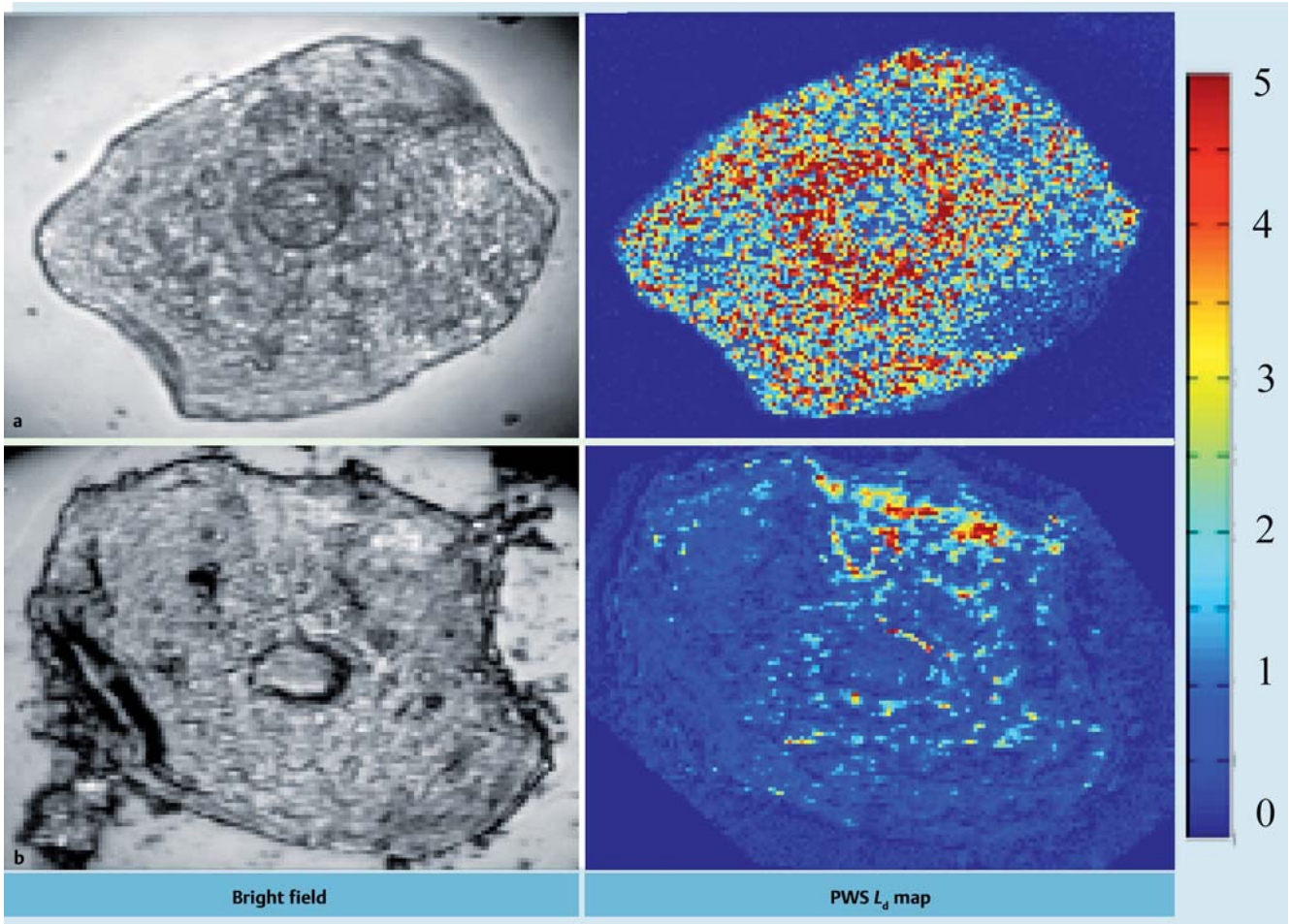

Fig. 1 Bright field image and a corresponding color-coded map of disorder strength (L_d), as assessed by partial wave spectroscopy (PWS) for representative normal-looking cells from the proximal esophagus. **a** Patient with esophageal adenocarcinoma. **b** Control patient.

density [19, 20]. Thus, two key features of PWS are its sensitivity to subdiffractional length scales and its ability to quantify spatial variations of macromolecular density.

For each topological point in a cell, the distribution of intracellular structures is quantified by the disorder strength

$L_d = \sigma_n^{\alpha}\, lc^{\beta}$

where σ_n and lc are the SD and the correlation length of the spatial fluctuations of refractive index. In a cell, σ_n is defined by the inhomogeneity of macromolecular density and lc is the characteristic size of the intracellular structures. The constants α and β are defined by the experimental set-up: α is between 1 and 2 depending on the refractive index contrast with the medium on top of a cell relative to its internal fluctuations; β depends on the collection numerical aperture of the instrument. In the experimental set-up employed in the current study, $\alpha = 1$ and $\beta \sim 1$. The disorder strength for every position (x,y) of a single biological cell is measured with PWS (**Fig. 1**).

For each patient in the current study, the disorder strength of 25 randomly chosen cells per patient was quantified. The person taking the measurements was blinded to the diagnosis. The mean of the resulting disorder strength distribution was referred to as the patient's mean disorder strength. The disorder strength measurements were normalized with respect to their time- and site-matched controls due to minor modifications made in the protocol with regard to alcohol fixation time.

Statistical analysis

Comparisons of demographic characteristics across the four groups were performed using Fisher's exact test for categorical variables and analysis of variance for continuous variables. Associations between disorder strength and diagnostic group or demographic characteristics were determined by fitting linear regression models with disorder strength as the dependent variable. Pairwise comparisons of disorder strength between diagnostic groups were performed using a Bonferroni correction. Non-parametric Wilcoxon rank-sum tests were used for subgroup analyses (e.g. comparison of disorder strength based on proton pump inhibitor use or cancer type). In addition, the area under the receiver operating characteristic (ROC) curve was calculated as a measure of the ability of disorder strength to differentiate between cancer and DBE vs. NDBE and control. Sensitivity, specificity, and percent correctly classified were also determined for given cut-off points. A P value < 0.05 was considered to be statistically significant. Statistical analyses were performed using Stata Version 12 (StataCorp, College Station, Texas, USA).

Results

Samples of the proximal esophagus from 75 patients were analyzed (**Table 1**). The mean age of patients was 58.7 years and 28 patients (37 %) were female. A total of 15 patients had patho-

Table 1 Demographic characteristics by patient group.

	Controls n=32	Non-dysplastic Barrett's n=15	Dysplastic Barrett's n=13	Esophageal cancer n=15	*P* value
Age, mean ± SD, years	51.3 ± 16.8	67.3 ± 10.7	66.2 ± 15.8	59.5 ± 8.9	< 0.01
Male, n (%)	13 (41)	10 (67)	12 (92)	12 (80)	< 0.01
Caucasian, n (%)	22 (69)	14 (93)	11 (85)	14 (93)	0.14
Smoking history, n (%)					0.05
Never	22 (69)	5 (33)	5 (39)	4 (27)	
Past	7 (22)	9 (60)	6 (46)	9 (60)	
Current	3 (9)	1 (7)	2 (15)	2 (13)	
Family history of esophageal cancer, n (%)	3/28 (11)	1/10 (10)	1/12 (8)	0/13 (0)	0.78

logically confirmed EAC (3 Intramucosal cancer and 12 invasive cancer), 13 patients had DBE (8 high grade and 5 low grade), and 15 had NDBE. A total of 32 individuals acted as controls, most of whom had reflux symptoms.

There was evidence for differences in the normalized disorder strength across the four groups in a linear regression model (P= 0.005) (**Fig. 2**). Specifically, the mean normalized disorder strength in the proximal esophagus for patients with EAC was significantly higher than that of controls (mean ± SD 1.807 ± 0.731 vs. 1.011 ± 0.432, respectively; Bonferroni corrected P< 0.01). Patients with DBE also had higher disorder strength (1.645 ± 0.757) than controls (Bonferroni P< 0.01). Patients with EAC and DBE also had a higher disorder strength than those with NDBE (1.101 ± 0.387; Bonferroni corrected P< 0.01 and P= 0.07, respectively). Although patients with NDBE had a higher mean disorder strength than controls, this difference was not statistically significant (Bonferroni corrected P= 1.00).

Assessment of potential confounding factors found no significant association between demographic characteristics and disorder strength (**Table 2**). As a check of the robustness of the results, a linear regression model that controlled for age and sex was also fit and the conclusions remained the same (data not shown).

By employing the ROC curve in this preliminary study, it was anticipated that disorder strength may exhibit fairly good accuracy in the differentiation between patients with cancer or DBE and those without, with an area under the ROC curve of 0.816 (95 % confidence interval [CI] 0.704 – 0.928) (**Fig. 3**). A normalized disorder strength cut-off of 1.25 above the average control value produced a sensitivity of 82.1 % (95 %CI 63.1 % – 93.9 %), a specificity of 83.0 % (95 %CI 69.2 % – 92.4 %), and 82.7 % were correctly classified (95 %CI 72.2 % – 90.4 %).

There were no statistically significant differences in disorder strength between patients taking proton pump inhibitor therapy and those not on therapy (P= 0.52). Furthermore, there were no statistically significant differences in disorder strength between LGD and HGD within the DBE group or between superficial cancer and invasive cancer in the cancer group (P= 0.22 and P= 0.62, respectively).

In order to determine whether the field effect extended to the oropharngyeal mucosa, samples of buccal mucosa from 14 patients were analyzed (6 EAC and 8 control patients). There was no significant difference in the disorder strength between the two groups (1.284 ± 0.730 for cancer group and 1.001 ± 0.433 for controls; P= 0.38) (**Fig. 4**).

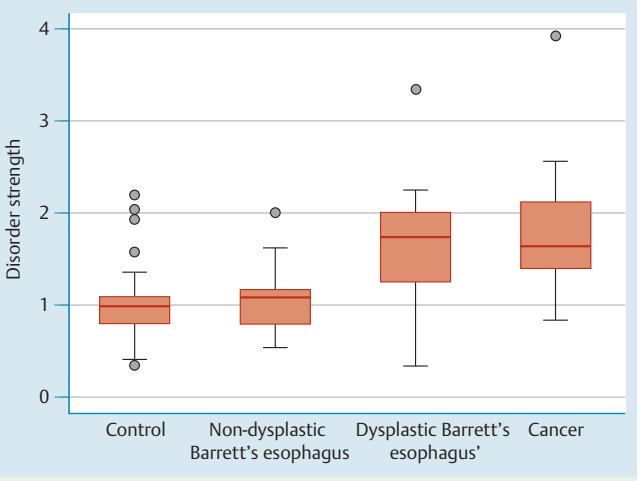

Fig. 2 Normalized disorder strength in the proximal esophagus of patients undergoing partial wave spectroscopy. There was a stepwise progression that correlated with histological stage. Furthermore, differences between dysplastic Barrett's esophagus and controls and between cancer and controls were statistically significant (P< 0.01 and P< 0.01, respectively). The median is designated as the line in middle of the box. The upper limit of the box refers to the 75th percentile and the lower limit of the box refers to the 25th percentile. Maximum and minimum values excluding outliers (shown as dots) are shown with the capped lines.

Table 2 Associations between demographic characteristics and disorder strength.

Variable	Regression coefficient	95 %CI	*P* value
Age (per 1-year increase)	0.005	– 0.004 to 0.015	0.29
Male	0.258	– 0.046 to 0.562	0.10
Caucasian	0.133	– 0.250 to 0.516	0.49
Smoking history			0.34
Never	Reference		
Past	0.232	– 0.083 to 0.547	
Current	0.153	– 0.350 to 0.656	
Family history of esophageal cancer	– 0.176	– 0.829 to 0.478	0.59

CI, confidence interval.

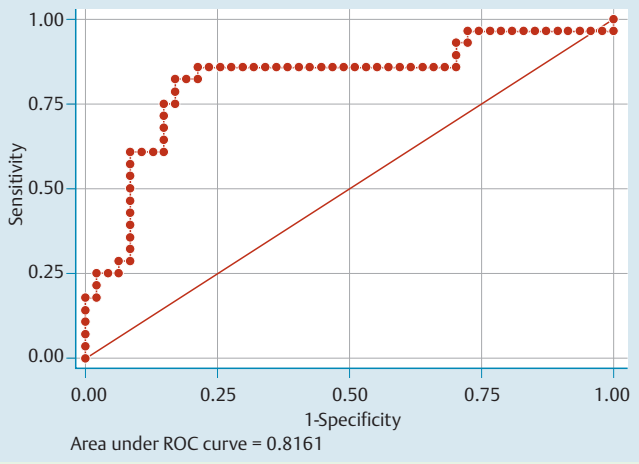

Fig. 3 Receiver operating characteristic (ROC) curve of partial wave spectroscopy for the detection of esophageal cancer or dysplastic Barrett's esophagus. The area under the ROC curve was 0.816 (95 % confidence interval 0.704 – 0.928) for the detection of cancer or dysplastic Barrett's esophagus.

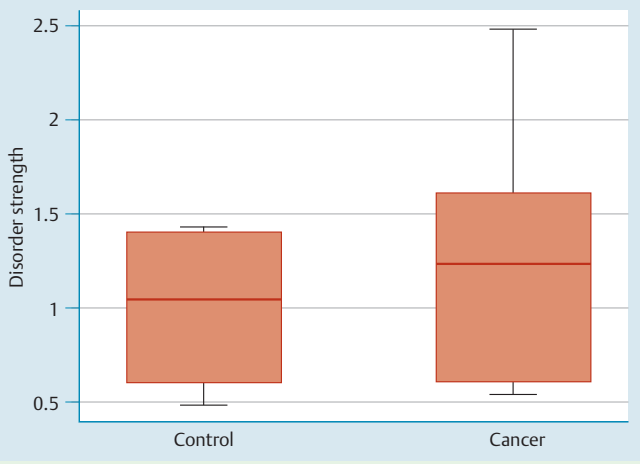

Fig. 4 Mean normalized disorder strength in the buccal mucosa between those patients with cancer and controls ($P = 0.38$). EAC, esophageal adenocarcinoma. The median is designated as the line in middle of the box. The upper limit of the box refers to the 75th percentile and the lower limit of the box refers to the 25th percentile. Maximum and minimum values excluding outliers (shown as dots) are shown with the capped lines.

Discussion

The current study demonstrated that PWS microscopy can determine differences at the nanoscale level in the normal proximal esophageal squamous mucosa between those patients who have progressed to esophageal cancer or Barrett's-associated neoplasia and control individuals.

Screening practices may become more targeted to select the individuals from the general population who may benefit from a screening endoscopy to look for Barrett's esophagus. Many biomarkers have been investigated in Barrett's-associated neoplasia, including the more promising markers with more evidence of aneuploidy, tetraploidy, and *p53 LOH* [21]. Ultimately, the most promising approach has often been speculated to be a combination of multiple markers as any single biomarker may not achieve independent prognostic value. Nanoarchitectural derangements that are not dependent on the detection of a specific protein, gene, or epigenetic event merit consideration as a promising approach to screening or surveillance. The current study demonstrated the proof of concept that derangements associated with Barrett's-associated neoplasia may be detected with PWS.

The current study was an exploratory study that warrants further investigation in biomarker development [22], with subsequent steps including additional case – control studies with larger sample sizes, prospective cohort studies, and randomized controlled trials. Future studies should also ensure better control for age and reflux history. Ultimately with these validation steps, PWS may have the potential to serve as a marker of field derangements at the nanoscale level to risk stratify patients in order to individualize surveillance or treatment strategy for individuals.

The biological phenomenon revealed in this study in the normal-appearing squamous epithelium may be further investigated in the following ways in future studies. An advantage that sampling of the proximal esophagus includes is that this may potentially be done without performing endoscopy and targeted biopsies. Collection devices that may be tethered with string to enable retrieval may be used to sample cells in the proximal esophagus as a possible non-endoscopic screening tool.

PWS findings in lung cancer studies have revealed nanoarchitectural and genetic differences between the buccal mucosa of patients with lung cancer and that in patients without lung cancer [16, 23, 24]. In the current study, preliminary studies were conducted to determine whether the extent of the field effect of esophageal cancer reached the buccal mucosa. No significant differences were found in the intracellular disorder strength of buccal mucosa between patients with and without esophageal cancer. The detection of nanoarchitectural changes in the proximal esophagus but not in the buccal mucosa is consistent with previous reports on field effect changes of distal esophageal cancer found in the proximal esophagus; such changes have not yet been documented in the buccal mucosa [11, 12]. Furthermore, the classic carcinogen for lung cancer – tobacco – would affect the buccal mucosa, whereas the classic mechanism for EAC – refluxed contents from the stomach – would not typically affect the buccal mucosa.

Limitations of this study include the small sample sizes within each group. Although statistically significant differences were demonstrated between groups (with and without controlling for age and sex), this small exploratory study did not control for age and sex differences during the patient selection phase. Future studies should incorporate age- and sex-matched controls. Although control patients mostly had reflux symptoms, either existing or historic, future studies may consider implementation of a more rigorous identification of reflux cases (i. e. confirmation of reflux esophagitis or acid exposure in the esophagus).

This study provides proof of concept that, as a result of the field effect of carcinogenesis, nanoarchitectural changes may be detectable in the normal squamous epithelial lining of the proximal esophagus in patients with Barrett's-associated neoplasia and esophageal cancer. Non-targeted samples from the proximal esophagus may be collected either with or without endoscopy. Further studies will explore the use of the field effect of esophageal carcinogenesis and PWS as a possible office-based screening tool that can be used to identify those patients with or without reflux in the general population who are not being referred for endoscopy for Barrett's screening. Potentially, further characterization of the potential of PWS in the proximal and distal esophagus may result in it being considered as a risk stratification tool if findings can be correlated with disease progression.

In conclusion, the current study established that by using PWS, nanostructural alterations can be detected in the adjacent histologically normal squamous epithelium (as a "field effect") of EAC and DBE. Advances in this technology and future validation studies may lead to the development of markers for screening or surveillance in Barrett's esophagus.

Competing interests: None.

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