Study Title: Office Based Screening Test for Barrett's Esophagus

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OFFICE BASED SCREENING TEST FOR BARRETT'S ESOPHAGUS

SPECIFIC AIMS:

Prospectively collected Surveillance Epidemiology and End Results (SEER) data indicate that the incidence of esophageal adenocarcinoma (EAC) has increased more than five-fold in the past three decades.[1-4] Over 10,000 cases are now diagnosed annually. The prognosis for patients with EAC is poor, with less than 20% of patients surviving beyond five years.[4,5]

Barrett's esophagus (BE), a pre-malignant metaplastic condition that is the only known precursor of EAC, is diagnosed only when patients undergo Esophagogastroduodenoscopy (aka Upper Endoscopy) (EGD). However, due to the high cost of EGD and the lack of a randomized controlled trial supporting its efficacy for preventing EAC associated mortality, endoscopy to screen for BE is not routinely recommended.[6,7] Thus, less than 5% of EACs are diagnosed in individuals with previously detected BE.[8] Most BE remains undetected. Clearly, to make an impact on the dismal prognosis of EAC, we need to first develop alternative methods for identifying BE that are less expensive than EGD and that can be widely accepted and adopted in a population at risk.

Dr. Sanford Markowitz's laboratory has discovered aberrant methylation of vimentin gene exon 1 DNA as a sensitive biomarker of gastrointestinal neoplasia.[19,25] Aberrant methylation of vimentin (mVIM) DNA can be detected in DNA extracted from BE in at least 90% of subjects with BE, making BE the most methylated VIM (mVIM) linked tissue change in the GI tract.[9] Moreover, in work featured on the cover of *Cancer Epidemiology, Biomarkers, and Prevention*, Markowitz and Chak showed that mVIM was easily detected in DNA from esophageal brushings of BE patients, suggesting the possibility of a non-endoscopic "molecular cytology" screening approach for BE screening and detection. The hypothesis of this proposal is that a sensitive molecular diagnostic test for BE, such as mVIM, in combination with a non-endoscopic brushing devices such as the capsule sponge proposed by Kadri et al,[10] will be a cost-effective method for BE screening. Our supplemental research proposal will support a clinical trial led by Drs. Markowitz and Chak to determine whether we can detect mVIM in BE patients using a novel non-endoscopic balloon based brushing device designed by Drs. Markowitz, Chak and Willis in collaboration with the industrial design group at Nottingham Spirk. The Specific Aims of this proposal are:

Specific Aim 1. *To determine acceptability of non-endoscopic balloon brushing of esophagus.*

Specific Aim 2. *To demonstrate successful performance of the mVIM assay in balloon brushings from subjects with and without BE.*

Specific Aim 3. *To examine molecular and histological factors that might lead to false negative and false positive mVIM assays in BE screening.*

Specific Aim 4. *To assay additional methylated DNA biomarkers that might improve the sensitivity and specificity of mVIM in balloon brushings.*

RESEARCH STRATEGY

Overview

To make an impact on the prevention and early detection of EAC the challenge is to first develop an alternative method for BE screening that is less costly and safer than EGD, while being more acceptable and accessible in the primary care setting. In preparation for a full scale screening study, we will first perform this pilot study at University Hospitals Case Medical Center and at the Cleveland Clinic. Together, these institutions see a large adult male population that is at increased risk for BE. A non-endoscopic method for detecting BE would allow screening in primary care clinics. We have demonstrated that aberrant mVIM can be detected in DNA extracted from endoscopic brushings in over 90% of subjects BE.[9] We now propose a pilot study to determine whether we can detect mVIM non-endoscopically by testing for mVIM in samples obtained from BE patients using a novel esophageal sampling device.

We wish to clarify that this pilot project only addresses the current challenges with BE screening. A non-endoscopic esophageal sampling device, such as the one proposed in this project, could potentially help with the surveillance and the assessment of BE recurrence after ablative therapies. These future applications will require the development of appropriate biomarkers that can be tested in prospective long-term full-scale studies.

SIGNIFICANCE

Early detection of BE requires the development of unsedated screening procedures that can be applied to an adult population at risk. -- The major limitations of the current guidelines that recommend sedated EGD in patients with multiple BE risk factors, refractory Gastroesophageal Reflux Disease (GERD), or alarm symptoms are clearly evident. This strategy fails to detect BE in asymptomatic subjects. It also fails to detect BE in patients whose symptoms are well controlled with either over the counter medications or physician-prescribed therapies. Thus, less than 5% of esophageal adenocarcinomas are diagnosed at an early stage in patients with previously detected BE.[8] Ablative non-surgical therapies that have been developed for BE with high grade dysplasia over the past decade11,12 will have little impact and the five year survival for esophageal adenocarcinomas will remain a dismal 15%13 unless we develop more effective programs for identifying BE in the general population.

Non-endoscopic detection of BE needs to improve. – Early studies of non-endoscopic balloon sampling of the esophagus relied on cytology, but were limited in their ability to obtain adequate material.[14] More recently, investigators from the UK reported on a device that can non-invasively assess for BE: an expandable Cytosponge in a dissolvable capsule that is swallowed. This sponge is tethered to a string, released in the stomach, and then retrieved, thus sampling the esophagus with a sensitivity of 73% and specificity of 94% for diagnosing BE.[10] This sponge method detects Barrett's epithelial cells by immunochemical staining using antibodies directed against TFF-3. It is not yet available in the United States. Robust molecular DNA marker(s) that could detect BE in esophageal brushings would be more preferable than current cytological techniques, because DNA tests can be automated and require far less material.

INNOVATION

Aberrant DNA methylation of vimentin (mVIM) is common in BE and Esophageal Adenocarcinoma – Aberrant methylation of CpG rich islands in DNA may lead to silencing of tumor suppressor genes and is a common event in many cancers. These methylated CpG islands can be identified in DNA extracted from various biospecimens (blood, urine, stool, etc.) and are potentially useful biomarkers for tumor detection. Aberrant methylation of the first exon of vimentin was initially discovered in Dr. Sanford Markowitz's laboratory in colon cancers and advanced colonic adenomas.[15] This soon led to the development of a robust stool DNA mVIM assay that was clinically adapted for colon cancer detection.[16] Subsequently, we found that mVIM was present in an even higher proportion of epithelial upper gastrointestinal tract tumors.[9] Specifically, mVIM was detected in 90% of non-dysplastic BE, dysplastic BE, and most esophageal adenocarcinomas (Figure 1).

mVIM can help diagnose BE in esophageal brushings. More recent research in the laboratory (unpublished) has concentrated on developing mVIM as a biomarker for detecting BE. Brushings were obtained from the distal esophagus/gastroesophageal junction and proximal squamous esophagus from 69 patients with normal esophagus or erosive esophagitis, 38 patients with non-dysplastic BE, 8 patients with low grade dysplasia (LGD), 10 with high grade dysplasia (HGD), and 63 patients with esophageal or junctional adenocarcinoma. mVIM > 1% was detected in DNA extracted from the distal esophagus brush in 88% of BE and adenocarcinoma patients and 14% of non-BE patients. The area under the ROC curve was 0.92, highly comparable to the AUC of 0.93 of the Exact panel (Cologuard assay) for colon cancer detection that recently won FDA

Fig. 2. ROC curve for methylated Vimentin positive assays in individuals with versus without BE and/or EAC. versus those without

approval. Interestingly, mVIM was also detected in the proximal esophagus in 41/113 (36%) smokers vs. 11/76 (14%) non-smokers, p = 0.0009, indicating that the presence of mVIM in the proximal esophagus is related to smoking. These studies suggest that mVIM can be used to detect BE and esophageal/junctional with a high sensitivity, but it will be necessary to use a device that samples the gastroesophageal junction and distal esophagus while avoiding contamination from the proximal esophagus.

Other methylation biomarkers are being developed. BETRNet Project 2 led by Dr. William Grady assayed genome wide methylation in a variety of BE and control tissues using the Illumina HM450 methylation array. Six promising candidate biomarkers were identified and validated by pyrosequencing assays. Technical and clinical validation studies were run with two of these markers (*ZNF793* and *B3GAT2*), and Methylight assays for m*ZNF793* and m*B3GAT2* were developed. These assays showed >95% sensitivity and specificity for detecting BE in a training set of esophagus cytology samples. Furthermore, *B3GAT2* and *ZNF793* methylation were also detectable in endoscopic brushings from the distal esophagus in four out of five BE cases tested.

Inflatable soft balloons can sample distal esophagus reliably and safely. – The concept of sampling the esophagus with an inflatable balloon was proposed nearly 20 years ago when Falk, et al. attempted to use a non-endoscopic inflatable balloon device to sample the esophagus.[14] Although the cytological material obtained in the Falk study was not able to identify dysplasia sensitively, the bio-material should be sufficiently adequate for assaying a molecular DNA marker such as mVIM. Nearly half the 63 patients in the Falk study found the balloon device as tolerable as regular EGD and there were no concerns regarding safety. The next step in the development of non-endoscopic esophageal sampling devices was the development of the Cytosponge in the UK. This tethered sponge inside a capsule has been found to be safe and tolerable in over 500 study participants.[10] However, this device is still susceptible to contamination from the proximal esophagus. Our experiments demonstrate it is important to avoid sampling the proximal esophagus because smoking causes methylation of VIM in the proximal squamous epithelium.

Figure 3. Balloon device

Balloon Device: For the present study, we propose to use an inflatable balloon device that incorporates next generation improvements compared to the device used by Falk, et al.[14] The new modified balloon device has been developed with the assistance of Nottingham Spirk, a nationally reputed industrial design firm. The balloon has been made thinner to be even more tolerable, and the surface has been modified to add soft bristles to improve cytological yield. In addition, the new balloon is also strategically designed to invert when it is deflated. This key feature will allow us to sample the distal esophagus tolerably, safely, and reliably while avoiding contamination from the proximal esophagus. The blow-molded balloon (Fig. 5) will be constructed of a typical biocompatible catheter material, which may be, but is not limited to, nylon or polyethylene. The size of the device is such that even if it is inadvertently swallowed it will pass safely through the digestive tract. The outer diameter of the delivery and inflation tubing will be approximately three mm, increasing to a maximum of approximately eight mm at

the balloon. The maximum inflated size of the balloon will be 18 mm. The balloon will be compliant and the pressure in the balloon will be less than the pressure generated in current esophageal sizing balloons that are safely inflated to diameters > 30 mm at pressures less than 0.25 atm. A pop-off relief valve will prevent over-pressurization of the balloon device during inflation. No esophageal perforations have been reported with high compliance low pressure esophageal balloon sizing in over 85,000 cases of esophageal ablation. The perforation rate is 0.01% with ablation balloons that are of size > 18 mm and inflated at higher pressures (radiofrequency ablation registry database, barrx.com). On preliminary testing, the size of the capsule balloon device appears to be a size that can be swallowed comfortably and safely. The balloon and capsule will be made of biocompatible material and of a size where, even if they are inadvertently swallowed or break, they will pass safely through the gastrointestinal tract. The device will be smaller in diameter and length than the FDA-approved small bowel capsule device that is designed to be swallowed and transit through the gastrointestinal tract. The manufacturer of the prototypes is ISO 14385 and it is FDA GMP certified.

Fig.4 Diagram of inverted balloon (left) and expanded balloon (right)

Fig 5. blow-molded balloon in pig esophagus

APPROACH

We now propose to combine a non-endoscopic esophageal sampling balloon device with the mVIM assay as a screening test to diagnose BE. This pilot study will test this strategy in a group of patients undergoing EGD at either University Hospitals Case Medical Center (UHCMC) or at the Cleveland Clinic (CCF). This study is being conducted at two institutions to enable the necessary recruitment and also to establish the infrastructure for a multi-center screening study. We will send de-identified samples to the Fred Hutchinson Cancer Center in Seattle, Washington, to be analyzed. If this study confirms that this approach is able to detect BE in at least 7 of 10 subjects with known BE, then for the BETRNet renewal we will propose a full scale screening study in subjects at risk for BE who have not had prior EGD.

We will recruit 40 subjects with BE who are undergoing EGD for surveillance and 80 subjects without BE who are undergoing EGD for other indications. Eligibility criteria for this study will be:

1. Adult patients at least > =18 years old who are undergoing clinically indicated EGD and can provide informed consent

2. No known coagulopathy, no known history of esophageal varices.

The study coordinator or a study investigator will approach patients scheduled for EGD in the endoscopy suites at UHCMC and CCF. At UHCMC, Dr. Amitabh Chak will act as Principal Investigator, with Dr. Sanford Markowitz as the Principal Investigator at the CCF and Dr. Joseph E. Willis acting as a Co-Investigator. Dr. Prashanti Thota will act as the Co-Investigator at the

Cleveland Clinic and will be assisted by Mary Oldenburgh, who will act as the RN Coordinator. The PI at Fred Hutchinson Cancer Center in Seattle, Washington, where de-identified samples will be analyzed, is Dr. William Grady. The study will be explained to the patient, who will then have the opportunity to ask questions. All subjects who consent to the research study will have esophageal balloon sampling prior to their EGD.

The technique for esophageal sampling will be similar to that used by Falk, et al.[14] The subject will be given topical pharyngeal anesthesia with benzocaine spray. The balloon will be swallowed and advanced to 50 cm. It will then be inflated fully with 15 cc air. It will be withdrawn until a tug is felt at the gastroesophageal junction (GEJ). Once the GEJ is located, 10 cc of air will be removed and the 5 cc balloon will be pulled back five cm to sample the distal esophagus. The balloon will then be completely deflated to cause inversion into its protective capsule, and will then be withdrawn. The balloon will be re-inflated outside the patient, and the obtained sample will be clipped with scissors into DNA buffer. The collected biospecimen will be stored frozen for later DNA extraction and assay of mVIM and other methylation markers. The vials will be labeled with a coded sample number (1, 2, 3, etc.). After all samples have been accrued, the coded samples will be assayed for mVIM in Dr. Markowitz's laboratory in Wolstein Building, CWRU, and other methylation markers will be assayed (m*ZNF793* and m*B3GAT2*) in Dr. Grady's laboratory at the Fred Hutchinson Cancer Research Center.

All patients will undergo standard EGD. Presence or absence of BE will be noted. All patients with suspected BE will have screen/surveillance biopsies to confirm diagnosis as per standard practice. These biopsy samples will also be tested for mVIM and other methylated markers. Endoscopic brushings will also be obtained from BE in cases and from the normal-appearing GEJ in controls.

Specific Aim 1. *To determine acceptability of non-endoscopic balloon brushing of esophagus.*

Subjects who agree to participate will complete a tolerability questionnaire that has been used in previous studies.[17] This questionnaire evaluates anxiety, pain, gagging, and choking using a Likert scale. The questionnaire also determines the subject's willingness to undergo the procedure in the future. All procedure-related complications will be recorded. A complication will be defined as an untoward and unplanned event that occurs during, or immediately after, the balloon procedure or administration of topical anesthetic. Data will also be collected regarding age, gender, race, indication for EGD, and findings on EGD. All patient identifiers will be stored by the study coordinator in a secure computer.

Analysis and Definitions of Tolerance: Descriptive statistics will be calculated for all tolerance variables (anxiety, pain, choking, gagging, and overall tolerance). The threshold for tolerability will be a score of < 3 on 10 point scale for symptoms elicited on the Tolerance Survey. The balloon screening procedure will be considered tolerable if < 15% of subjects who undergo the screening procedure score all symptom questions < 3 and at least 80% are willing to undergo the procedure again if needed.

Specific Aim 2. *To assay mVIM in balloon brushings from subjects with and without BE.*

DNA from esophageal samples. Following esophageal sampling, balloons will be clipped into nuclease free 1.5ml cryo-safe tubes with buffer and immediately frozen. The tubes will be labeled with codes that are de-identified to the laboratory. DNA will be purified using QIAamp DNA micro kit (QIAGEN) according to the immediately for bisulfite conversion, or frozen at -80°C until use.

Bisulfite conversion of the genomic DNA and real-time MS-PCR assay. To create a template for methylation-specific PCR, DNA samples will be subjected to bisulfite conversion and purified using an Epitect kit (QIAGEN), according to the manufacturer's protocol. 1-100ng of bisulfite-converted DNA at a concentration of 0.2-the real-time MS-PCR assay. To normalize input DNA amounts, a companion real-time PCR assay will be designed against bisulfite converted Actin gene sequences that lack CpG dinucleotides and so are not affected by DNA methylation. The assay for Actin was designed to generate an amplification product of the same size as the assay for methylated VIM. For both ACTIN and VIM real-time PCR, a mixture of DNA from four colon cancer cell lines that are each fully methylated across the VIM CpG island will be used to generate a dilution standard curve that will be run with all real-time assays and used as part of data analysis in BioRad CFX manager software to convert the Ct values into ng DNA amounts. VIM methylation will be calculated as a percentage ratio of the amount of methylated DNA measured by VIM qPCR, divided by total bisulfite-converted DNA amount in the sample, as measured by Actin qPCR. The real-time MS-volume uses Light Cycler PCR master mix (Roche) with 400nM each primer and 200nM probe.[9] Amplifications will be done in 96-well plates in CFX96 Real-Time System (BioRad) under the following conditions: 95°C for 10 min, followed by 50 cycles of 30sec at 95°C, and 60 sec at primer-specific annealing/extension temperature.

Sample Size and Analysis: Descriptive statistics will be generated for demographic variables and other variables of interest. For this pilot study, we have selected to assay 40 subjects with BE and 80 subjects without BE. We will compare patient endoscopic and pathologic findings relating to the diagnosis of BE with mVIM analyses of corresponding esophageal balloon derived samples. This is a sufficient sample size to give us a measure of tolerance and to also determine if mVIM is able to detect BE in at least 70% of subjects with known BE. The presence of at least 1% of methylated VIM in DNA extracted from the esophageal sampling balloon will be considered a positive assay. The sensitivity and specificity of the balloon mVIM assay for detecting BE will be calculated. Balloon device mVIM assays will be correlated with endoscopic brushing mVIM assays by calculating a Pearson's correlation coefficient. This method will not be considered adequate if it is not tolerable or if it is not able to detect mVIM in at least 80% of subjects with BE with 90% specificity. The balloon device will be considered acceptable if the correlation coefficient with endoscopic brushings is at least 0.9.

Specific Aim 3. *To examine molecular and histological factors that might lead to false negative and false positive VIM assays in BE screening.*

In our preliminary study of DNA extracted from esophageal brushings obtained during endoscopy, mVIM had an 88% sensitivity and 86% specificity. To try and understand potential factors that lead to false negative mVIM tests in 12% of subjects with BE or EAC and false

positive mVIM tests in 14% of subjects without BE, we will perform molecular and histological studies.

We have formalin fixed paraffin embedded (FFPE) and snap frozen tissue available from 6 BE and 8 HGD/EAC cases that were mVIM negative on their esophageal brushings. We will select an additional 6 BE and 8 HGD/EAC samples that were mVIM positive as controls. Dr. Joseph E. Willis, an expert gastrointestinal pathologist at CWRU, will blindly grade the FFPE histology of these 14 mVIM brushing negative and 14 mVIM brushing positive samples for the presence of goblet cells (percent of specialized intestinal metaplasia – 0- <25%, 25 - <50%, 50 - < 75%, 75 – 100%), percentage cardia type mucosa, intestinalization (cancer cases), and cancer grade (cancer cases only). Since the sample size is small, this will be a descriptive "hypothesis generating" analysis, where we will explore any differences in histo-morphology between BE and EAC that are mVIM negative and mVIM positive on brushings.

To confirm that the mVIM negative brushings are indeed associated with low levels of VIM methylation, we will also extract DNA from the mVIM brushing negative BE and EAC tissues. mVIM will be assayed in the tissues and levels of VIM methylation in the biopsy tissue will be correlated with levels of VIM methylation in the DNA from esophageal brushings. A finding that low levels of mVIM in brushings are correlated with low levels of mVIM in biopsy material will indicate the need for additional methylation markers (see Aim 4) to improve the sensitivity of methylated markers for BE screening. A finding that low mVIM esophageal brushings can occur in the presence of high levels of mVIM methylation in tissues will reinforce the need for minimized contamination when performing balloon sampling.

The esophageal brushing study we performed found mVIM > 1% in 10 of 69 subjects who had no evident BE. Three of these ten false positive cases occurred in the presence of high mVIM from the proximal esophagus in smokers. Our sampling device has been designed to invert the balloon into a protective hood to minimize proximal esophageal contamination. Other explanations for positive mVIM at the GE junction might be the presence of intestinal metaplasia in a normal appearing cardia or the presence of VIM methylation in gastric metaplasia of the esophagus.

Indeed, the very definition of BE is debated. Glandular metaplasia of the distal esophagus is the primary etiology of most esophageal adenocarcinomas. In the US and as used in this proposal, the working definition of BE includes the presence of intestinal metaplasia (IM) occurring in variable proportion of gastric-type columnar mucosa in the distal esophagus. In the United Kingdom, glandular metaplasia of the esophagus linked to increased risk of EAC, i.e. BE, is defined solely by the presence of gastric-type columnar mucosa in the esophagus regardless of the presence or absence of IM. Previous studies have identified molecular features of intestinal differentiation in gastric-type columnar mucosa adjacent to IM.[18] Another important glandular abnormality is IM of the cardia the presumed precursor lesion of gastroesophageal junctional adenocarcinoma. We propose to identify the presence or absence of mVIM in each of these glandular abnormalities using our extensive biorepository of clinically acquired formalin fixed paraffin embedded [FFPE] biopsies. We have previously demonstrated our ability to analyze for mVIM on FFPE specimens.[9]

Dr. Willis will obtain 20 de-identified FFPE blocks from distal esophageal samples with only glandular metaplasia without IM; 20 samples of glandular metaplasia with extensive IM [defined as greater than 80% of cells are IM]; 20 samples of glandular metaplasia with small amounts of IM [defined as less than 10% of cells are IM]; 20 samples from the cardia without IM; and 20 samples from the cardia with IM from the CWRU GI Cancer Tissue Archive. Histology will be reviewed to confirm the diagnosis. DNA will be extracted from FFPE tissues and assayed for level of mVIM as described above.

We will determine the proportion of samples that show mVIM > 1% for each defined histology. These results will especially be useful in determining whether it will be necessary to obtain a biopsy from a normal appearing gastroesophageal junction when we design our full scale BE screening study.

Specific Aim 4. *To assay additional methylated DNA biomarkers that might improve the sensitivity and specificity of VIM in balloon brushings.*

Studies by Dr. Grady's laboratory have already validated m*B3GAT2* and m*ZNF793* as methylation markers for BE screening and found that these markers are also detectable in esophageal brushings. We will assay the methylation of these and any other screening markers that validate through our BETRNet pipeline (Drs. Markowitz' and Grady's laboratories) using DNA samples from the 40 BE and 80 non-BE cases that undergo balloon sampling in Aim 1.

Analysis: Since mVIM is a robust biomarker with an AUC of 0.92 that has already been tested clinically, we will first determine if any of the new biomarkers increase the sensitivity and specificity for BE screening when combined with mVIM. We will then perform an independent analysis, where we will model an independent panel of biomarkers for BE screening. Using Receiver Operating Characteristics (ROC) analysis, we will rank biomarkers based on the highest Area Under the Curve (AUC) values. This type of analysis provides a continuum of sensitivity and specificity across different threshold settings for the biomarker. Rankings based on AUC values will be established for ability to discriminate BE from normal esophagus. Thirdly, a rank list will determined with respect to the overall prevalence of significant methylation in primary BE cases, and the absence of DNA methylation in normal cases. The rank lists will be cross-referenced to generate a panel of the most promising biomarkers, with an emphasis on those that have strong predictive ability (i.e. no detectable methylation in normal cases). We will select the panel with the highest sensitivity and specificity for further assessment in a full scale screening trial.

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