

# Barrett's Esophagus and Esophageal Adenocarcinoma Biomarkers **A C**

William M. Grady<sup>1,2</sup>, Ming Yu<sup>2</sup>, Sanford D. Markowitz<sup>3</sup>, and Amitabh Chak<sup>4</sup>



## ABSTRACT

Esophageal adenocarcinoma is a major cause of cancer-related morbidity and mortality in Western countries. The incidences of esophageal adenocarcinoma and its precursor Barrett's esophagus have increased substantially in the last four decades. Current care guidelines recommend that endoscopy be used for the early detection and monitoring of patients with Barrett's esophagus; however, the efficacy of this approach is unclear. To prevent the increasing morbidity and mortality from esophageal adenocarcinoma, there is a tremendous need for early detection and surveillance biomarker assays that are accurate, low-cost, and clinically feasible to implement. The last decade has seen remarkable advances in the development of minimally invasive

molecular biomarkers, an effort led in large part by the Early Detection Research Network (EDRN). Advances in multi-omics analysis, the development of swallowable cytology collection devices, and emerging technology have led to promising assays that are likely to be implemented into clinical care in the next decade. In this review, an updated overview of the molecular pathology of Barrett's esophagus and esophageal adenocarcinoma and emerging molecular biomarker assays, as well as the role of EDRN in biomarker discovery and validation, will be discussed.

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## Introduction

Symptoms of long-standing gastroesophageal reflux disease (GERD) have historically been the main clinical features used to identify people at risk of having Barrett's esophagus, who are then advised to undergo endoscopic assessment. It is clear that many people with Barrett's esophagus have no history of GERD, which is one of several major reasons behind the lack of success of current Barrett's esophagus–screening and surveillance programs for preventing esophageal adenocarcinoma (1). With regards to strategies to identify Barrett's esophagus at high risk of progressing to esophageal adenocarcinoma, the presence or absence of Barrett's esophagus with or without dysplasia on histologic review is currently the only biomarker used clinically for risk stratification and directing treatment (2, 3). This dearth of well-studied biomarkers and the reliance on reflux symptoms and endoscopic findings of dysplasia had led to what Reid called the “paradox” of Barrett's esophagus management (4). In this paradox, Reid notes several frustrating epidemiologic facts: (i) a large number of individuals with Barrett's esophagus are asymptomatic, (ii) nearly 50% develop esophageal adenocarcinoma without associated GERD symptoms, (iii) 95% of esophageal adenocarcinomas arise without a prior diagnosis of Barrett's esophagus, and (iv) nearly 80% of esophageal adenocarcinoma arise without a prior diagnosis of GERD (1, 4).

Furthermore, the vast number of people with Barrett's esophagus detected by endoscopy will not progress to esophageal adenocarcinoma and instead will die of unrelated causes, which reflects the late age of occurrence of most esophageal adenocarcinomas. In fact, the majority of people with Barrett's esophagus are more likely to die from complications of cardiac disease than from esophageal adenocarcinoma (5). With these insights, several areas of active research in molecular biology are underway to resolve the “paradox” of Barrett's esophagus and are likely to lead to more effective approaches to identifying and managing those patients with Barrett's esophagus. Through efforts of Early Detection Research Network (EDRN) investigators as well as others, a number of promising markers have been identified; however, currently there are only a limited number of biomarkers to precisely identify patients with Barrett's esophagus and those at high risk of progression to esophageal adenocarcinoma.

With recent advances in genomics (i.e., next-generation sequencing), epigenomics, proteomics, and microarray technology, many potential diagnostic and prognostic molecular biomarkers have been identified at the level of DNA, RNA, and individual proteins. These technologies have been used to characterize the molecular profiles of Barrett's esophagus and esophageal adenocarcinoma and to advance our understanding of the molecular alterations that define Barrett's esophagus, dysplasia, and esophageal adenocarcinoma. They have also led to the recent identification of promising biomarkers that will likely impact clinical care in the next decade, if not sooner.

## Barrett's Esophagus and Esophageal Adenocarcinoma Overview

Barrett's esophagus, which is specialized small intestinal metaplastic epithelium of the esophagus, is a precursor to esophageal adenocarcinoma, a cancer that has increased dramatically in the last 40 years. Most, if not all, esophageal adenocarcinoma originates in Barrett's esophagus. Esophageal adenocarcinoma appears to arise via a metaplasia–dysplasia–carcinoma sequence whereby Barrett's

<sup>1</sup>Department of Medicine, University of Washington School of Medicine, Seattle, Washington. <sup>2</sup>Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington. <sup>3</sup>Oncology Division, Case Western Reserve University and University Hospitals Cleveland Medical Center, Cleveland, Ohio. <sup>4</sup>Gastroenterology Division, Case Western Reserve University and University Hospitals Cleveland Medical Center, Cleveland, Ohio.

**Corresponding Author:** William M. Grady, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, D4-100, Seattle, WA 98109. Phone: 206-667-1107; Fax: 206-667-2917; E-mail: wgrady@fredhutch.org

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metaplasia can progress to low-grade dysplasia (LGD), then high-grade dysplasia (HGD) before becoming intramucosal carcinoma and finally invasive carcinoma (6, 7). Advances in endoscopic therapy over the past two decades have made it feasible to intervene at the dysplastic stage to prevent the progression to esophageal adenocarcinoma without resorting to esophagectomy, which has substantial postoperative long-term morbidity.

### Classes of Molecular Alterations in Barrett's Esophagus and Esophageal Adenocarcinoma: Characterization of Frequency of Alterations and Their Biology

#### Genomic alterations

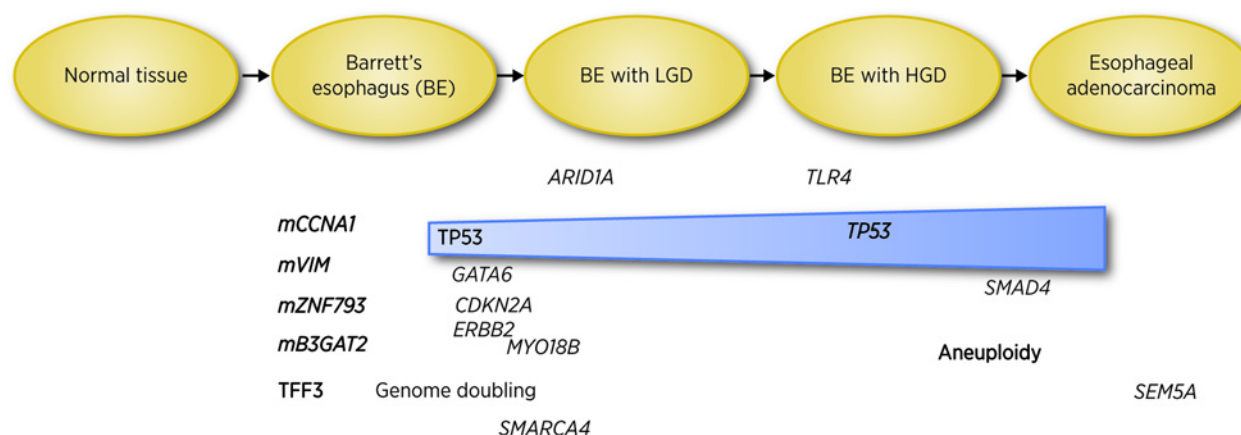
A comprehensive analysis of somatic mutations in esophageal adenocarcinoma using whole-exome sequencing and whole-genome sequencing has been recently performed (ref. 8; Fig. 1). The investigators analyzed 149 esophageal adenocarcinoma tumor-normal matched fresh-frozen samples and identified a series of significantly mutated genes, including "classical" tumor-driver genes, such as *TP53*, *CDKN2A*, *SMAD4*, *ARID1A*, and *PIK3CA*, as well as new candidate driver genes, such as *SPG20*, *TLR4*, *ELMO1*, and *DOCK2*, among others. Chromosomal instability and copy-number alterations have been found in Barrett's esophagus and esophageal adenocarcinoma (1, 9). Paulson and colleagues (9) identified 9p loss encompassing the *p16/CDKN2A* locus in Barrett's esophagus, HGD, and esophageal adenocarcinoma cases; losses of chromosome 5q, 13q, and 18q in HGD and esophageal adenocarcinoma; and high-level amplification at *ERBB2* on chromosome 17q in esophageal adenocarcinoma.

More recent studies of Barrett's esophagus have revealed an unexpected number of pathogenic variants of a number of oncogenes and tumor-suppressor genes in approximately 10%–20% of Barrett's esophagus cases. An analysis of 25 matched cases of Barrett's esophagus and esophageal adenocarcinoma using directed exome next-generation sequencing revealed common tumor-suppressor gene mutations, with few oncogene mutations and genomic alterations

present (10). This study found that mutations in *TP53* and *SMAD4* were the most prevalent mutations in Barrett's esophagus and that two pathways of Barrett's esophagus progression appeared to be present. One pathway involves *TP53* mutations and genomic doubling and may lead to the majority of esophageal adenocarcinoma cases (>60%), whereas the other pathway involves the serial accumulation of mutations and is enriched for lesions with *SMAD4* and *CDKN2A* alterations. Mutation analyses have shown that with the exception of *TP53* and *SMAD4*, genes altered in Barrett's esophagus and esophageal adenocarcinoma do not display differential mutation rates between Barrett's esophagus and esophageal adenocarcinoma, even for *bona fide* tumor suppressors such as *CDKN2A* (30%) and *ARID1A* (15%), and others including *KMT2D*, *MYO18B*, *UNC13C*, *FBXW7*, *ATM*, *FAT2*, *LRP1B*, *SMARCA4*, etc. (all <5%). *TP53* mutations have been found in less than 5% of non-dysplastic Barrett's esophagus, whereas 70% of cases of HGD and esophageal adenocarcinoma were *TP53* mutant (10, 11). These results suggest that genetic alterations beyond *TP53* and *SMAD4* are not likely to yield clinically useful biomarkers for Barrett's esophagus risk stratification.

#### Epigenomic alterations

Epigenetic alterations, such as DNA hypermethylation in the promoter regions of genes, have also been identified in Barrett's esophagus and esophageal adenocarcinoma and are found in the majority of Barrett's esophagus and esophageal adenocarcinoma cases (refs. 12–14; Fig. 1). EDRN-funded studies by Kaz and colleagues (15) have shown that factors, including aging, smoking, and obesity, may play a role in the formation of these epigenetic alterations (16). Hypermethylated genes include known tumor-suppressor genes, such as *APC*, *CDKN2A* (*p16INK4a*), *RUNX3*, *MGMT*, *CDH1*, and *SFRP* family members among others (12). A subset of the hypermethylated genes are believed to play a driver role in driving the formation of esophageal adenocarcinoma, but many appear to be Barrett's esophagus and esophageal adenocarcinoma-specific passenger alterations (12, 14). Aberrant methylation of classic tumor-suppressor genes such as *CDKN2A* and *MGMT* has been correlated with loss of mRNA and protein expression in the metaplasia–dysplasia–carcinoma sequence of Barrett's esophagus (17, 18). Recently, through EDRN support, Yu and colleagues (14) identified four methylation subtypes of esophageal



**Figure 1.** Representation of the Barrett's esophagus to esophageal adenocarcinoma progression sequence and accompanying genomic and epigenomic alterations. The alterations shown are a representative, but not complete list of genes affected by mutation, amplification, or aberrant methylation. Those alterations shown to be candidate biomarkers are in bold text.

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adenocarcinoma and Barrett's esophagus through genome-wide DNA methylation profiling. The high-methylator (HM) subtype had more activating events in *ERBB2* and a higher global mutation load, compared with the other subtypes. In addition, this study uncovered a novel molecular mechanism by which esophageal adenocarcinoma cells activate the oncogenic ERBB2/EGFR signaling pathway via epigenetically silencing the tyrosine phosphatase non-receptor 13 (*PTPN13*), specifically in the HM subtype.

Of relevance to biomarker discovery, a large number of genes and loci have been identified as high-frequency targets of aberrant methylation in Barrett's esophagus and esophageal adenocarcinoma (14). Although the functional significance of these methylated genes is still not clear, these DNA methylation events have proved to be highly promising as biomarkers of Barrett's esophagus, as discussed below. In summary, the published studies to date suggest that aberrant DNA methylation is a common molecular mechanism that mediates the development of esophageal cancer and that aberrantly methylated genes and loci are very promising biomarkers for Barrett's esophagus and esophageal adenocarcinoma.

#### MicroRNA alterations

miRNA/miRs are small noncoding RNA molecules of approximately 20 nucleotides that appear to play important roles in diverse cellular processes during carcinogenesis. There is a continually growing number of studies focusing on the potential biological roles of miRNA/miRs in esophageal cancer development (19, 20). For example, several studies have shown overexpression of miR-192 during Barrett's esophagus→esophageal adenocarcinoma progression (13). miR-192 is a downstream target of TP53 and plays a tumor-suppressor role through cell-cycle arrest (21). From a clinical perspective, an interesting finding is that altered miRNAs can be detected in the blood of patients with esophageal cancer (22), which suggests that they may be readily accessible molecular markers for early detection and monitoring chemotherapeutic responsiveness (23). However, the studies published to date have often produced conflicting results, likely secondary in large part to the wide-spread use of non-validated analysis methods that are not robust and reproducible. This lack of consistency among studies has substantially limited progress in this area of research and in the use of miRNA/miRs as biomarkers.

#### Protein alterations

In addition to alterations in genomic DNA, the epigenome, and miRNA/miR expression, aberrant protein expression has also been noted in Barrett's esophagus and esophageal adenocarcinoma. These aberrantly expressed proteins for the most part play an unclear role in the pathogenesis of Barrett's esophagus and esophageal adenocarcinoma, but they have been shown to be useful as biomarkers for Barrett's esophagus. Immunostain assays for two proteins, TFF3 and TP53, have been shown to be robust markers for non-dysplastic Barrett's esophagus and advanced dysplasia, respectively (24), and are discussed in more detail in a following section.

## Novel Methods for Barrett's Esophagus Screening and Surveillance

### Barrett's esophagus–screening markers

Genetic and epigenetic alterations occurring in Barrett's esophagus and early-stage esophageal cancer have the potential to be used as early-detection biomarkers. As noted above, candidate early-detection markers include somatic mutations, aberrantly

methylated genes, overexpressed miRNAs, as well as deregulated proteins.

### Somatic variants, deletions, and rearrangements

As noted earlier, gene mutations arise in the Barrett's esophagus→esophageal adenocarcinoma progression sequence and affect a substantially greater proportion of Barrett's esophagus with dysplasia and esophageal adenocarcinoma cases compared with non-dysplastic Barrett's esophagus cases. This class of molecular alteration was the first type studied in Barrett's esophagus and esophageal adenocarcinoma and has shown potential to be a class of biomarkers for Barrett's esophagus and esophageal adenocarcinoma (25). Chromosomal instability and copy-number alterations have been found in Barrett's esophagus and esophageal adenocarcinoma (1). Paulson and colleagues (9) identified 9p loss encompassing the *p16/CDKN2A* locus in Barrett's esophagus, HGD, and esophageal adenocarcinoma cases; losses on chromosomes 5q, 13q and 18q in HGD and esophageal adenocarcinoma; and high-level amplification at *ERBB2* on chromosome 17q in esophageal adenocarcinoma. In addition, genome-wide association studies have identified common variants that are associated with genetic susceptibility to Barrett's esophagus (26). Dong and colleagues (27) developed a polygenic risk score (PRS) using genomic variants and found individuals in the highest quartile of risk, based on genetic factors (PRS), had a 2-fold higher risk of Barrett's esophagus [OR, 2.22; 95% confidence interval (CI), 1.89–2.60] or esophageal adenocarcinoma (OR, 2.46; 95% CI, 2.07–2.92) than individuals in the lowest quartile of risk. When they combined data on demographic or lifestyle factors with data on GERD symptoms, they identified patients with Barrett's esophagus with an AUC of 0.793 and patients with esophageal adenocarcinoma with an AUC of 0.745 (27).

A subset of these candidate genomic DNA-based biomarkers have been assessed in case–control clinical studies, including abnormal DNA ploidy, alterations in DNA copy number [based on fluorescent *in situ* hybridization (FISH); refs. 28–31], gene mutations, loss of heterozygosity (LOH) of specific DNA loci (32), and measurements of clonal diversity in the Barrett's esophagus tissue (33). These molecular alterations have been shown in early-phase studies to serve as adjunctive markers to delineate the degree of dysplasia (e.g., use of FISH probes for *C-MYC* to confirm HGD or carcinoma; ref. 30) or to further risk stratify patients at greatest risk for progression to esophageal adenocarcinoma (e.g., loss of ploidy associates with a 38.7% increased relative risk of developing esophageal adenocarcinoma; ref. 28). Unfortunately, genetic alterations do not appear to be of value as Barrett's esophagus–screening biomarkers because of their low prevalence in Barrett's esophagus cases. In contrast, *TP53* mutations appear to have potential to be esophageal adenocarcinoma–screening biomarkers (11).

### Aberrantly methylated genes

Aberrantly methylated genes and DNA loci have been shown to be robust biomarkers for use in cancer care and prevention for a variety of cancers. Studies largely conducted by EDNRN investigators over the last 3 years have shown methylated DNA biomarkers to be the most promising class of Barrett's esophagus and esophageal adenocarcinoma biomarkers to date. Through the EDNRN, Moinova and colleagues (34) recently demonstrated that methylated *VIM* has a high sensitivity for detecting esophageal adenocarcinomas and Barrett's esophagus, and that it even exceeded the robust sensitivity for detecting colon cancer that they had already shown. The identification of methylated *VIM* DNA as a biomarker of Barrett's esophagus suggested

the potential for biomarker-based early detection of Barrett's esophagus and esophageal adenocarcinoma. This finding prompted Moinova and colleagues to develop a "molecular cytology" assay for methylated *VIM* in DNA samples from esophageal cytology brushings obtained during endoscopies of 322 individuals, divided into training and validation cohorts (35). The assay showed 91% sensitivity for detecting Barrett's esophagus, Barrett's esophagus with dysplasia, and esophageal adenocarcinoma at 93% specificity, with essentially identical results obtained in both the training and validation cohorts (35). To further improve performance of a Barrett's esophagus detection assay, they conducted a genome-wide analysis of DNA methylation in Barrett's esophagus tissue samples using reduced representation bisulfite sequencing and found methylated *CCNA1* DNA as a second Barrett's esophagus biomarker with performance in both training and validation cohorts similar to methylated *VIM* (35). When combined, the two-marker panel of methylated *VIM* and methylated *CCNA1* DNAs detected 95% of Barrett's esophagus, Barrett's esophagus and dysplasia, and esophageal adenocarcinoma cases at 91% specificity, including detecting 96% of Barrett's esophagus with dysplasia and 96% of esophageal adenocarcinoma (35).

To advance this biomarker panel toward a practical method for early Barrett's esophagus detection, Moinova and colleagues (35) developed and engineered a swallowable balloon-based device for obtaining targeted non-endoscopic brushings of the distal esophagus. To use the device, patients swallow a vitamin pill-sized capsule that contains the balloon and is attached to a thin silicone catheter connected to an external syringe. After passage into the stomach, the balloon is inflated with air injected through the catheter and then pulled back into the esophagus to brush the gastro-intestinal junction plus a 6-cm length of distal esophagus. Removal of air via the catheter inverts the balloon back into the capsule, thereby protecting the distal esophagus sample from further dilution and from potential contamination by methylated DNA present in the proximal esophagus and oropharynx. In a clinical trial of 86 subjects, examination with the balloon could be completed in less than 5 minutes with 95% of subjects stating they would recommend the procedure to others (35). Analysis for methylated *VIM* and methylated *CCNA1* of DNA samples extracted from the balloon demonstrated 90% sensitivity for detecting non-dysplastic Barrett's esophagus with 92% specificity (35) providing practical demonstration of a biomarker-based approach for detecting this asymptomatic precursor for esophageal adenocarcinoma. In 2019, Lucid Diagnostics received FDA approval for commercial manufacture of the balloon device under the tradename EsoCheck. The combination of the balloon device and the methylated DNA panel is currently being further validated by testing in a nationwide multicenter clinical trial as well as undergoing commercial development under the tradename EsoGuard.

Additional promising Barrett's esophagus markers have been identified and validated by others, including the laboratory of W. M. Grady, an EDRN investigator. Yu and colleagues (36) discovered two genes, *B3GAT2* and *ZNF793*, that are aberrantly methylated in Barrett's esophagus. Clinical validation studies confirmed that *B3GAT2* and *ZNF793* methylation levels were significantly higher in Barrett's esophagus samples (median 32.5% and 33.1%, respectively) than in control tissues (median 2.29% and 2.52%, respectively;  $P < 0.0001$  for both genes) and that gene-specific MethyLight assays could accurately detect Barrett's esophagus ( $P < 0.0001$  for both) in endoscopic brushing samples with *mZNF793*

having a sensitivity of 70% and specificity of 100% for Barrett's esophagus. These markers show promise to further improve the performance of a methylated gene panel for Barrett's esophagus screening.

In addition to the Esocheck device, other swallowable cytology collection devices are being assessed and currently being evaluated for use in Barrett's esophagus-screening assays, such as the "Cytosponge" and "Esophacap" devices, which are both swallowed capsules that degrade in the stomach to release a sponge tethered to a string (38, 39). Unlike the Esocheck device, these devices sample the entire esophagus and oropharynx, which increases the potential of impairing biomarker performance. Similar to the Esocheck device, they capture esophageal cells that can later be analyzed for particular molecular changes associated with Barrett's esophagus and/or dysplasia (39). Using a Cytosponge-based assay, Chettouh and colleagues (40) discovered and assessed hypermethylated *TFPI2*, *TWIST1*, *ZNF345*, and *ZNF569* as potential Barrett's esophagus-screening markers. Methylated *TFPI2* was shown to achieve the best sensitivity in both the pilot and validation Cytosponge cohorts (85% and 79%, respectively, AUC 0.88).

In summary, these studies have established that methylated DNA has emerged as a promising new biomarker class that will enable practical non-endoscopic screening and early detection of Barrett's esophagus, an approach with potential to reduce the steadily increasing mortality from esophageal adenocarcinoma. These developments have been vigorously supported by the NCI EDRN program and embody the EDRN's vision for the potential of biomarkers to enable early cancer detection and to reduce cancer-related mortality.

#### Protein alterations

A number of proteins are differentially expressed in Barrett's esophagus and esophageal adenocarcinoma compared with the normal esophagus. Lao-Sirieix and colleagues (38) surveyed three publicly available microarray datasets to identify putative biomarkers present in Barrett's esophagus but absent from normal esophagus and gastric mucosa. They identified TFF3 and DDC as the most promising candidate biomarkers for Barrett's esophagus. Validation studies demonstrated TFF3 as the highest-performing biomarker. The authors consequently developed an immunostain assay based on TFF3 in esophageal cytology samples for Barrett's esophagus. In a case-control clinical study, they found that TFF3-positive cytology samples collected using the Cytosponge had a reasonable sensitivity (87%) and specificity (92%) for detection of Barrett's esophagus segments greater than 3 cm in length (38). This TFF3 Barrett's esophagus detection assay is being further assessed in the actively enrolling BEST-3 clinical trial (see below).

### Barrett's Esophagus Biomarker Clinical Trials

There are currently a number of clinical trials assessing different combinations of these swallowable cytology collection devices and selected biomarkers assays for the early detection of Barrett's esophagus, Barrett's esophagus with dysplasia, and esophageal adenocarcinoma (Table 1). Trials that are actively recruiting at the time of this publication include the following:

1. NCT02560623; Highly discriminant methylated DNA markers for the non-endoscopic detection of Barrett's esophagus. Primary site: Mayo Clinic, principal investigator Prasad G. Iyer.



**Table 1.** Validated Barrett’s esophagus early detection markers.

Biomarker	Method	Study design	AUC	Sensitivity	Specificity	Ref.
TFF3	IHC (Cytosponge)	BEST-2: Case-control (N = 1,110)		80%	92%	(60)
<i>mVIM</i> and <i>mCCNA1</i>	bsNSG (Esocheck device)	Case-control Validation cohort (N = 86)		90%	92%	(35)
<i>mB3GAT2</i>	methyLight PCR (endoscopic brushings)	Case-control Validation cohort (N = 66)	0.95	80%	86%	(36)
<i>mZNF793</i>	methyLight PCR (endoscopic brushings)	Case-control Validation cohort (N = 66)	0.96	80%	93%	(36)
<i>mTFPI2</i>	methyLight PCR (Cytosponge)	Case-control Validation cohort (N = 278)	0.88 (0.84–0.91)	82%	96%	(40)
<i>mTWIST1</i>	methyLight PCR (Cytosponge)	Case-control validation cohort (N = 278)	0.81 (0.77–0.86)	70%	93%	(40)

Note: The table summarizes Barrett’s esophagus biomarkers that have been evaluated in clinical cohorts. Abbreviations: bsNSG, bisulfite next-generation sequencing; IHC, immunohistochemistry.

- NCT00288119; Genetic determinants of Barrett’s esophagus and esophageal adenocarcinoma (FBE). Primary site: Case Western Reserve University, principal investigator Amitabh Chak (supported by the NCI, EDRN and BETRNet).
- NCT02890979; Swallowable sponge cell sampling device and next-generation sequencing in detecting esophageal cancer in patients with LGD or HGD, Barrett’s esophagus, or GERD. Primary center: Oregon Health Sciences University, principal investigator James Dolan.
- Offman J, Muldrew B, O’Donovan M, Debiram-Beecham I, Pesola F, Kaimi I, et al. Barrett’s oESophagus trial 3 (BEST3): study protocol for a randomised controlled trial comparing the Cytosponge-TFF3 test with usual care to facilitate the diagnosis of oesophageal pre-cancer in primary care patients with chronic acid reflux. *BMC Cancer* 2018;18:784.

**Barrett’s Esophagus Surveillance and Risk Prediction Markers**

Barrett’s esophagus is associated with approximately 4× increased risk of esophageal adenocarcinoma, which has led to the recommendation that patients with Barrett’s esophagus undergo regular endoscopic surveillance (3). However, only 0.1%–0.3% of people with Barrett’s esophagus will progress to HGD or esophageal adenocarcinoma each year; thus, a biomarker (or biomarker panel) would be of great clinical utility if it can accurately risk stratify high-risk patients with Barrett’s esophagus who are likely to progress from those low-risk patients with Barrett’s esophagus who are unlikely to develop esophageal adenocarcinoma (41). Such a marker could potentially spare the great majority of individuals with a diagnosis of Barrett’s esophagus from the cost, inconvenience, and risks of regular endoscopic surveillance. Being placed in a “low-risk” group might also reduce the feelings of anxiety about developing esophageal adenocarcinoma that have been shown to be associated with a diagnosis of Barrett’s esophagus (42).

The search for accurate risk stratification markers for Barrett’s esophagus is an area of intense investigation that has led to identification of a number of promising risk biomarkers. To date, none of these markers have proven adequate to be used in the clinical setting, although immunostaining assays for p53 and aneuploidy appear highly promising (3).

**Methylated DNA markers**

In a retrospective study, EDRN investigator S. Meltzer compared patients with Barrett’s esophagus who progressed to HGD or esophageal adenocarcinoma with those who did not, using hypermethylated *CDKN2A* (OR, 1.74; 95% CI, 1.33–2.20), *RUNX3* (OR, 1.80; 95% CI, 1.08–2.81), and *HPPI* (OR, 1.77; 95% CI, 1.06–2.81), which were associated with an increased risk of progression. Age, Barrett’s esophagus SL, and hypermethylation of other genes (*TIMP3*, *APC*, or *CRBP1*) were not found to be independent risk factors (42). A follow-up study using these same epigenetic markers in combination with three clinical parameters (gender, Barrett’s esophagus SL, and pathologic assessment) demonstrated this multi-parameter method could stratify patients with Barrett’s esophagus into high, intermediate, and low risk for progression to HGD or esophageal adenocarcinoma. This tissue-based assay has not been adopted into routine clinical use to date (43). In a later iteration of this approach, this risk assessment tool was expanded to include additional genes previously shown to be hypermethylated in Barrett’s esophagus and/or esophageal adenocarcinoma, most of which have been described in the previous section, to generate an eight-marker risk-of-progression panel. In a retrospective analysis of 145 non-progressors and 50 progressors, this panel predicted progression with a sensitivity of approximately 50% when the specificity was set at 90% (44). None of these candidates have advanced to phase III or IV biomarker trials (Table 2).

**MicroRNA alterations**

miRNA/miRs are a class of small noncoding RNAs that are often abnormally expressed in cancer. Expression profiles of miRNAs have been used to characterize molecular subtypes of cancers, and as prognostic and predictive markers for certain cancers. By employing high-throughput techniques, such as microarrays and next-generation sequencing, a number of recent studies have identified candidate miRNAs as markers of malignant progression of Barrett’s esophagus.

In studies of Barrett’s esophagus, dysplasia, and esophageal adenocarcinoma, miR-196a, miR-192, miR-194, miR-106b, miR-25, mi-93, let-7c, miR-200, miR-203, miR-205, miR-192, miR-215, and miR-196b have shown incremental increases in expression with each step of progression from normal esophagus to metaplasia to dysplasia and carcinoma (45–49). In a pilot phase 2 cross-sectional study, Bansal and colleagues (50) compared miRNA expression signatures in metaplasia

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**Table 2.** Candidate Barrett's esophagus risk stratification markers.

Biomarker	Study design	Sample size	Outcome
Abnormal DNA ploidy, 9pLOH, 17pLOH (33)	Prospective cohort	N = 243	RR = 38.7 (95% CI, 10.8–138.5)
Aneuploidy, tetraploidy (61)	Retrospective analysis	N = 322	RR = 11 (95% CI, 5.5–21)
LOH by FISH: 17p13.1 (62)	Retrospective analysis of surveillance cohort	N = 151	5% of NDBE 9% of LGD 46% of HGD
CNA and LOH by FISH: 8q24, 9p21, 17q11.2, 20q13.2 (63)	Prospective	N = 138	LGD: sens 70%, spec 89% HGD: sens 84%, spec 93% EAC: sens 94%, spec 93%
Hypermethylation of CDKN2A, RUNX3, HPPI (64)	Retrospective and longitudinal	N = 53	CDKN2A OR, 1.74 RUNX3 OR, 1.8 HPPI OR, 1.77
Jin methylated gene panel (65)	Retrospective, multicenter, double-blinded	N = 50 progressors N = 145 non-progressors	AUC = 0.843 at 2 years AUC = 0.829 at 4 years
TissueCypher (55)	Case-control multicenter	N = 145 non-progressors, N = 45 progressors	OR, 9.4 high vs. low risk (95% CI, 2.65–33.28) OR, 2.35 intermediate vs. low risk (95% CI, 0.66–8.41)

Abbreviations: CI, confidence interval; CNA, copy number alteration; EAC, esophageal adenocarcinoma; FISH, fluorescent *in situ* hybridization; HDG, high-grade dysplasia; LGD, low-grade dysplasia; LOH, loss of heterozygosity; NDBE, non-dysplastic Barrett's esophagus.

tissues from patients with Barrett's esophagus with or without dysplasia/cancer, and identified miR-15b, -203, and -21 as being discriminatory between patients with Barrett's esophagus with and without dysplasia/cancer, which suggested their potential utility for risk stratification. More recently, Leidner and colleagues (51) comprehensively characterized miRNA alterations during progressive stages of esophageal adenocarcinoma. They found 26 miRNAs that are highly and frequently deregulated in Barrett's esophagus and esophageal adenocarcinoma when compared with paired normal esophageal squamous tissue (51). They identified miR-31 and -375 as potential markers of progression during early and late stages of tumorigenesis, respectively. In an independent study, Wu and colleagues (20) confirmed miR-375 as a miRNA being downregulated exclusively in cancers, supporting its role as a marker of cancer progression in Barrett's esophagus.

Although significant progress has been made in characterizing miRNA alterations in Barrett's esophagus and esophageal adenocarcinoma, there are still substantial limitations of the existing data, most notably being the lack of a consensus miRNA signature of cancer risk across the different studies. This is likely a consequence of studies with small sample sizes, inherent variations among study populations, differing methods for detecting miRNAs, and cellular heterogeneity in Barrett's esophagus and esophageal adenocarcinoma. In addition, progress in this field has been impeded by the poor reproducibility of study results, which reflects the lack of robust and reliable detection methods and the lack of sufficient attention to the confounding effects of preanalytical variables. Furthermore, the expression level differences between disease and normal states are often suboptimal for development of robust biomarkers. These limitations will need to be overcome for miRNA/miR-based biomarkers to be clinically useful.

**Clonal alterations and LOH**

Maley and colleagues (33) have conducted numerous studies describing the relationship between clonal diversity and clonal expansions and the risk of Barrett's esophagus progression. One prospective study of 268 patients with Barrett's esophagus evaluated

whether clonal expansions during the progression of Barrett's esophagus leads to homogenous cell populations or results in clonal diversity. The authors found that patients with greater clonal diversity had greater risk of progression to esophageal adenocarcinoma ( $P < 0.001$ ). In a follow-up study, this group compared clonal diversity in 79 Barrett's esophagus progressors and 169 non-progressors over 20,425 person-months of follow-up, finding that non-progressors had types of chromosomal instability (small localized deletions involving fragile sites and 9p loss/copy neutral LOH) that generated relatively little genetic diversity (52). Meanwhile, individuals that progressed to esophageal adenocarcinoma developed chromosome instability with initial gains and losses, genomic diversity, and selection of somatic chromosomal alterations followed by catastrophic genome doublings. These data suggest that molecular testing to assess risk of progression in Barrett's esophagus may need to incorporate assessment of structural genomic alterations and multiple foci of Barrett's esophagus from individual patients and that such an assay could then be used as a risk prediction biomarker.

In another study that was a retrospective cohort study of high-risk patients who had a history of biopsy-confirmed HGD without esophageal adenocarcinoma, endoscopic brushing specimen were analyzed by FISH probes targeting 8q24 (*MYC*), 9p21 (*CDKN2A*), 17q12 (*ERBB2*), and 20q13 (*ZNF217*). The presence of polysomy was associated with a significantly higher risk of developing esophageal adenocarcinoma within 2 years (14.2%), compared with patients with a non-polysomic FISH result (1.4%,  $P < 0.001$ ; ref. 31).

**Altered TP53 expression and TP53 mutation**

Altered TP53 tissue expression is the most promising risk stratification biomarker to date and has near-term potential to be used in clinical care. A large number of case-control studies have suggested that overexpression of TP53 in Barrett's esophagus tissue indicates an increased risk for esophageal adenocarcinoma, especially for Barrett's esophagus with LGD.

In the last 10 years, a series of studies by investigators at Erasmus MC University Medical Center found that increased TP53

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expression in Barrett's esophagus, determined by IHC, preceded development of HGD/esophageal adenocarcinoma by several years and that TP53 expression was an important risk factor for HGD/esophageal adenocarcinoma with an HR of 6.5 (95% CI, 2.5–17.1; refs. 53, 54). In the largest study to date, TP53 immunostaining ( $N = 635$  patients, 12,000 biopsies), overexpression and complete loss significantly associated with the risk of neoplastic progression after adjusting for age, gender, Barrett's esophagus length, and esophagitis [RR, 5.6 (95% CI, 3.1–10.3) and RR, 14.0 (95% CI, 5.3–37.2), respectively]. However, only 49% of patients who progressed had aberrant TP53 immunostaining, which significantly limits its potential clinical utility. Furthermore, in a nested case-control study by an independent group of investigators that used a registry of patients with Barrett's esophagus in Ireland, TP53 protein overexpression did not predict progression in a multivariate analysis (29).

Currently, TP53 is not routinely recommended for risk stratification, but the British Society of Gastroenterology does have a grade B recommendation to test TP53 by IHC to clarify an equivocal histologic diagnosis of dysplasia (3). The low sensitivity of this assay and concerns about reproducibility of the assay are still major concerns about its use in the clinic.

#### TissueCypher

The TissueCypher (Cernostics) is a quantitative, multiplexed biomarker-imaging assay. It uses 14 epithelial and stromal biomarkers (K20, Ki-67, BETA-CATENIN, p16INK4a, AMACR, p53, HER2/neu, CDX-2, CD68, NF-kBp65, COX-2, HIF1a, CD45RO, and CD1a). In a multi-institutional case-control study, a 3-tier 15-feature classifier was identified in a training set ( $N = 183$ ) and tested in a validation set ( $N = 183$ ). The classifier stratified patients into low-, intermediate-, and high-risk classes [HR, 9.42; 95% CI, 4.6–19.24 (high-risk vs. low-risk);  $P < 0.0001$ ]. It also provided independent prognostic information that outperformed predictions based on pathology analysis, segment length, age, sex, or TP53 overexpression (55). This assay is a promising tissue-based prediction assay for progression to HGD or esophageal adenocarcinoma but requires further evaluation in prospective studies in appropriate populations to determine its clinical utility.

#### Blood-Based Assays

Blood, stool, or saliva biomarker-based assays, in principal, are an ideal screening or surveillance method given the easy access of samples and safety of collection. A number of candidate blood-based biomarkers, including methylated DNA, circulating miRNA/miRs, metabolite panels, and peptides, have been identified in small, retrospective, *in vitro* and non-human trials, although to date none have

been evaluated in prospective clinical trials (59, 60, 62). Most recently, in a proof-of-principle study, Qin and colleagues (59) demonstrated that a 5-methylated DNA biomarker panel (*FER1L4*, *ZNF671*, *ST8SIA1*, *TBX15*, *ARHGEF4*) used in a plasma-based assay achieved an AUC of 0.93 (95% CI, 0.89–0.96) on best-fit and 0.81 (95% CI, 0.75–0.88) on cross-validation. At 91% specificity, the panel detected 74% of esophageal cancer (esophageal adenocarcinoma and esophageal squamous cell cancer) overall, and 43%, 64%, 77%, and 92% of stages I, II, III, and IV, respectively.

#### Conclusions

Remarkable advances in early detection assays and technologies have occurred over the last decade. The most promising class of biomarkers for Barrett's esophagus early detection is based on aberrantly methylated DNA. The EDRN has played a central role in the discovery and development of Barrett's esophagus early detection assays that use non-endoscopic minimally invasive devices. Progress in the development of minimally invasive biomarkers for esophageal adenocarcinoma and for predicting the risk for esophageal adenocarcinoma in patients with Barrett's esophagus has also been made but no markers to date have been validated for use in clinical care. There is a great promise that the next decade will see the advent of this next generation of Barrett's esophagus-screening assays in the clinic and that well-validated assays for the detection of esophageal adenocarcinoma will be determined. The EDRN and its investigators have played and will undoubtedly continue to play a central role in Barrett's esophagus and esophageal adenocarcinoma biomarker research.

#### Authors' Disclosures

W.M. Grady is an advisory board member for Freenome and SEngine, a consultant for Guardant Health and DiaCarta, and reports receiving a commercial research grant from Janssen. S.D. Markowitz reports founders stock from, is a consultant and board member for, has licensed patents with, and a commercial research grant from Lucid Diagnostics and has ownership interest (including patents) in Lucid Diagnostics and Exact Sciences. A. Chak is an adviser for Lucid Diagnostics and a consultant for Interpace. No disclosures were reported by the other author.

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