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# Barretts Esophagus and Esophageal Adenocarcinoma Biomarkers

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## Abstract

Esophageal adenocarcinoma (EAC) is a major cause of cancer related morbidity and mortality in Western countries. The incidences of EAC and its precursor Barretts esophagus (BE) 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 BE, however, the efficacy of this approach is unclear. In order to prevent the increasing morbidity and mortality from EAC, 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 BE and EAC and emerging molecular biomarker assays, as well as the role of EDRN in biomarker discovery and validation, will be discussed.

### Keywords

Barrett’s esophagus; esophageal squamous cell carcinoma; esophageal adenocarcinoma; mutation; epigenetic; DNA methylation; microRNA; proteomics; biomarkers

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Conflicts of Interest:

W. M. Grady is an advisory board member for Freenome, Guardant Health, and SEngine. He is an investigator in a clinical trial sponsored by Janssen Pharmaceuticals. S. D. Markowitz receives income related to patents licensed to Exact Sciences, has founders shares and stock options in LucidDx, serves on the board of directors of LucidDx, serves as a consultant to LucidDx, has sponsored research with LucidDx, and has a royalty interest in patents licensed to LucidDx. A. Chak is an equity holder and advisor for Lucid Diagnostics, consultant for Interpace Diagnostics, nd receives research support from C2Therapeutics/Pentax Inc.

## Introduction

Symptoms of long-standing gastroesophageal reflux disease (GERD) have historically been the main clinical features used to identify people at risk of having Barretts esophagus (BE), who are then advised to undergo endoscopic assessment. It is clear that many people with BE have no history of GERD, which is one of several major reasons behind the lack of success of current BE screening and surveillance programs for preventing esophageal adenocarcinoma (EAC)[1]. With regards to strategies to identify BE at high risk of progressing to EAC, the presence or absence of BE 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 BE management.[4] In this paradox, Reid notes several frustrating epidemiologic facts: 1) a large number of individuals with BE are asymptomatic, 2) nearly 50% develop EAC without associated GERD symptoms, 3) 95% of EACs arise without a prior diagnosis of BE, and 4) nearly 80% of EAC arise without a prior diagnosis of GERD[1,4]. Furthermore, the vast number of people with BE detected by endoscopy will not progress to EAC and instead will die of unrelated causes, which reflects the late age of occurrence of most EACs. In fact, the majority of people with BE are more likely to die from complications of cardiac disease than from EAC[5]. With these insights, several areas of active research in molecular biology are underway to resolve the "paradox" of BE and are likely to lead to more effective approaches to identifying and managing those patients with BE. Through efforts of 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 BE and those at high-risk of progression to EAC.

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 BE and EAC and to advance our understanding of the molecular alterations that define BE, dysplasia, and EAC. They have also led to the recent identification of promising biomarkers that will likely impact clinical care in the next decade, if not sooner.

## BE and EAC Overview

Barretts esophagus (BE), which is specialized small intestinal metaplastic epithelium of the esophagus, is a precursor to esophageal adenocarcinoma (EAC), a cancer that has increased dramatically in the last 40 years. Most, if not all, EAC originates in Barrett's esophagus (BE). EAC appears to arise via a metaplasia-dysplasia-carcinoma sequence whereby Barrett's metaplasia can progress to low-grade dysplasia, then high-grade dysplasia 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 EAC without resorting to esophagectomy, which has substantial post-operative long-term morbidity.

## Classes of Molecular Alterations in BE and EAC: Characterization of Frequency of Alterations and Their Biology

### Genomic Alterations

A comprehensive analysis of somatic mutations in EAC using whole-exome sequencing and whole-genome sequencing has been recently performed[8]. (See Figure 1). The investigators analyzed 149 EAC 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 BE and EAC[1, 9]. Paulson et al identified 9p loss encompassing the *p16/CDKN2A* locus in BE, high-grade dysplasia (HGD) and EAC cases, losses of chromosome 5q, 13q and 18q in HGD and EAC, and high-level amplification at *ERBB2* on chromosome 17q in EAC[10].

More recent studies of BE have revealed an unexpected number of pathogenic variants of a number of oncogenes and tumor suppressor genes in approximately 10–20% of BE cases. An analysis of 25 matched cases of BE and EAC using directed exome next generation sequencing revealed common tumor suppressor gene mutations, with few oncogene mutations and genomic alterations present[11]. This study found that mutations in *TP53* and *SMAD4* were the most prevalent mutations in BE and that two pathways of BE progression appeared to be present. One pathway involves *TP53* mutations and genomic doubling and may lead to the majority of EAC cases (>60%), while 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 BE and EAC do not display differential mutation rates between BE and EAC, 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 nondysplastic BE; whereas, 70% of cases of HGD and EAC were *TP53* mutant[11, 12] These results suggest that genetic alterations beyond *TP53* and *SMAD4* are not likely to yield clinically useful biomarkers for BE risk stratification.

### Epigenomic Alterations

Epigenetic alterations, such as DNA hypermethylation in the promoter regions of genes, have also been identified in BE and EAC and are found in the majority of BE and EAC cases[13–15]. (See Figure 1.) EDRN funded studies by Grady and colleagues have shown that factors including aging smoking and obesity may play a role in the formation of these epigenetic alterations[16, 17]. Hypermethylated genes include known tumor-suppressor genes, such as *APC, CDKN2A (p16INK4a), RUNX3, MGMT, CDH1*, and *SFRP* family members among others[13]. A subset of the hypermethylated genes are believed to play a driver role in driving the formation of EAC, but many appear to be BE and EAC specific passenger alterations[13, 15]. 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[18, 19]. Recently, through

EDRN support, Yu et al identified four methylation subtypes of EAC and BE through genome-wide DNA methylation profiling[15]. The high methylator subtype (HM) had more activating events in *ERBB2* and a higher global mutation loa, compared to the other subtypes. In addition, this study uncovered a novel molecular mechanism by which EAC 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 BE and EAC[15]. 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 BE, as discussed below. In summary, he published studies to date suggest 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 BE and EAC.

### MicroRNA alterations

MicroRNAs 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 microRNAs (miRNA/miR) in esophageal cancer development[20, 21]. For example, several studies have shown overexpression of miR-192 during BE→EAC progression[14]. miR-192 is a downstream target of TP53 and plays a tumor-suppressor role through cell-cycle arrest[22]. From a clinical perspective, an interesting finding is that altered miRNAs can be detected in the blood of patients with esophageal cancer[23], which suggests they may be readily accessible molecular markers for early detection and monitoring chemotherapeutic responsiveness.[24]. 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 microRNAs as biomarkers,

### Protein alterations

In addition to alterations in genomic DNA, the epigenome, and microRNA expression, aberrant protein expression has also been noted in BE and EAC. These aberrantly expressed proteins for the most part play an unclear role in the pathogenesis of BE and EAC, but they have been shown to be useful as biomarkers for BE. Immunostain assays for two proteins, TFF3 and TP53, have been shown to robust markers for non-dysplastic BE and advanced dysplasia, respectively[25] and are discussed in more detail in a following section.

## Novel Methods for BE screening and surveillance

### BE screening markers

Genetic and epigenetic alterations occurring in Barrett esophagus (BE) 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 BE→EAC progression sequence and affect a substantially greater proportion of BE with dysplasia and EAC cases compared to non-dysplastic BE cases. This class of molecular alteration was the first type studied in BE and EAC and has shown potential to be biomarkers for BE and EAC[26]. Chromosomal instability and copy number alterations have been found in BE and EAC[1]. Paulson et al identified 9p loss encompassing the *p16/CDKN2A* locus in BE, high-grade dysplasia (HGD) and EAC cases and losses on chromosome 5q, 13q and 18q in HGD and EAC, and high-level amplification at *ERBB2* on chromosome 17q in EAC[10]. In addition, genome-wide association studies have identified common variants that are associated with genetic susceptibility to BE[27]. Dong et al developed a polygenic risk score using genomic variants and found Individuals in the highest quartile of risk, based on genetic factors (PRS), had a 2-fold higher risk of BE (odds ratio, 2.22; 95% confidence interval, 1.89–2.60) or EAC (odds ratio, 2.46; 95% confidence interval, 2.07–2.92) than individual in the lowest quartile of risk. When they combined data on demographic or lifestyle factors with data on GERD symptoms identified patients with BE with an AUC of 0.793 and patients with EAC with an AUC of 0.745[28].

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 fluorescence in-situ hybridization (FISH))[29–32], gene mutations, loss of heterozygosity of specific DNA loci[33], and measurements of clonal diversity in the BE tissue[34]. 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[31]) or to further risk stratify patients at greatest risk for progression to EAC (e.g., loss of ploidy associates with a 38.7% increased relative risk of developing EAC[29]). Unfortunately, genetic alterations do not appear to be of value as BE screening biomarkers because of their lowprevalence in BE cases. In contrast, *TP53* mutations appear to have potential to be EAC screening biomarkers[12].

**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 EDRN investigators over the last 3 years have shown methylated DNA biomarkers to be the most promising class of BE and EAC biomarkers to date. Through the EDRN, Markowitz and colleagues recently demonstrated that methylated *VIM* has a high sensitivity for detecting esophageal adenocarcinomas (EAC) and Barrett's esophagus (BE), and that it even exceeded the robust sensitivity for detecting colon cancer that they had already shown[35]. The identification of methylated *VIM* DNA as a biomarker of BE suggested the potential for biomarker based early detection of BE and EAC. This finding prompted Markowitz's team 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[36]. The assay showed 91% sensitivity for detecting BE, BE with dysplasia, and EAC at 93% specificity, with essentially identical

results obtained in both the training and validation cohorts[36] To further improve performance of a BE detection assay, they conducted a genome-wide analysis of DNA methylation in BE tissue samples using reduced representation bisulfite sequencing and found methylated *CCNA1* DNA as a second BE biomarker with performance in both training and validation cohorts similar to methylated *VIM*[36]. When combined, the two-marker panel of methylated *VIM* and methylated *CCNA1* DNAs detected 95% of BE, BE and dysplasia and EAC cases at 91% specificity, including detecting 96% of BE with dysplasia and 96% of EAC[36].

To advance this biomarker panel toward a practical method for early BE detection, Markowitz, Chak, and colleagues developed and engineered a swallowable balloon based device for obtaining targeted non-endoscopic brushings of the distal esophagus[36]. 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[36]. Analysis for methylated *VIM* and methylated *CCNA1* of DNA samples extracted from the balloon demonstrated 90% sensitivity for detecting non-dysplastic BE with 92% specificity[36] providing practical demonstration of a biomarker based approach for detecting this asymptomatic precursor for EAC. 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 multi-center clinical trial as well as undergoing commercial development under the tradename EsoGuard.

Additional promising BE markers have been identified and validated by others, including the lab of William Grady, an EDRN investigator. Yu et al discovered two genes, *B3GAT2* and *ZNF793*, that are aberrantly methylated in BE. Clinical validation studies confirmed *B3GAT2* and *ZNF793* methylation levels were significantly higher in BE 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 BE ($P < 0.0001$ for both) in endoscopic brushing samples with *mZNF793* having a sensitivity of 70% and specificity of 100% for BE[37]. These markers show promise to further improve the performance of a methylated gene panel for BE screening.

In addition to the Esocheck device, other swallowable cytology collection devices are being assessed and currently being evaluated for use in BE 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[38–40]. Unlike the Esocheck device, these devices sample the entire esophagus and oropharynx, which increases the potential impairing biomarker performance. Similar to the Esocheck device, they capture esophageal

cells which can later be analyzed for particular molecular changes associated with BE and/or dysplasia[40]. Using a Cytosponge based assay, Chettouh et al discovered and assessed hypermethylated *TFPI2, TWIST1, ZNF345* and ZNF569 as potential BE 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)[41].

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 BE, an approach with potential to reduce the steadily increasing mortality from EAC. 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 mortality.

**Protein alterations—**A number of proteins are differentially expressed in BE and EAC compared to the normal esophagus. Lao-Sirieix and colleagues surveyed three publicly available microarray datasets to identify putative biomarkers present in BE but absent from normal esophagus and gastric mucosa[39]. They identified TFF3 and DDC as the most promising candidate biomarkers for BE. Validation studies demonstrated TFF3 as the highest performing biomarker. The authors consequently developed an immunostain assay based on TFF3 in esophageal cytology samples for BE. 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 BE segments greater than 3cm in length[39]. This TFF3 BE detection assay is being furthered assessed in the actively enrolling BEST-3 clinical trial. (See below)

## BE 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 BE, BE with dysplasia and EAC. (See 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.

2) NCT00288119; Genetic Determinants of Barrett's Esophagus and Esophageal Adenocarcinoma (FBE); Primary site: Case Western Reserve University, Principal Investigator: A.Chak (supported by the NCI, EDRN and BETRNet)

3) NCT02890979; Swallowable Sponge Cell Sampling Device and Next Generation Sequencing in Detecting Esophageal Cancer in Patients With Low or High Grade Dysplasia, Barrett Esophagus, or Gastroesophageal Reflux Disease; Primary center: Oregon Health Sciences University, PI: James Dolan

4) BEST 3: BMC Cancer. 2018 Aug 3;18(1):784. doi: 10.1186/s12885-018-4664-3.

## BE surveillance and risk prediction markers

BE is associated with approximately 4X increased risk of EAC, which has led to the recommendation that patients with BE undergo regular endoscopic surveillance[3]. However, only 0.1–0.3% of people with Barrett's esophagus will progress to high-grade dysplasia or EAC each year, thus, a biomarker (or biomarker panel) would be of great clinical utility if it can accurately risk stratify high risk patients with BE who are likely to progress from those low risk BE patients who are unlikely to develop EAC[43]. Such a marker could potentially spare the great majority of individuals with a diagnosis of BE 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 EAC that have been shown to be associated with a diagnosis of BE[44].

The search for accurate risk stratification markers for BE 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 Steve Meltzer compared BE patients who progressed to HGD or EAC to 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 *HPP1* (OR 1.77, 95% CI 1.06 – 2.81), which were associated with an increased risk of progression. Age, BE segment length, and hypermethylation of other genes (*TIMP3, APC*, or *CRBP1*) were not found to be independent risk factors[45]. A follow-up study using these same epigenetic markers in combination with three clinical parameters (gender, BE segment length (SL), and pathologic assessment) demonstrated this multi-parameter method could stratify BE patients into high, intermediate, and low risk for progression to HGD or EAC. This tissue based assay has not been adopted into routine clinical use to date[46]. In a later iteration of this approach, this risk assessment tool was expanded to include additional genes previously shown to be hypermethylated in BE and/or EAC, 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 nonprogressors and 50 progressors, this panel predicted progression with a sensitivity of ~50% when the specificity was set at 90%[47]. None of these candidates have advanced to phase III or IV biomarker trials.

### MicroRNA alterations

MicroRNAs (miRNA/miR) are a class of small non-coding 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 BE.

In studies of Barretts esophagus, dysplasia and esophageal adenocarcinoma, miR-196a, miR-192, miR-194, miR-106b, miR-25, mi-93, let-7c, miR-200,miR-203, −205, −192, −215, and miR-196b have shown incremental increases in expression with each step of progression from normal esophagus to metaplasia to dysplasia and carcinoma[4849.505153.54]. In a pilot phase 2 cross-sectional study, Bansal and colleagues compared miRNA expression signatures in metaplasia tissues from BE patients with or without dysplasia/cancer, and identified miR-15b, −203, and −21 as being discriminatory between BE patients with and without dysplasia/cancer, which suggested their potential utility for risk stratification[52]. More recently, Leidner and colleagues comprehensively characterized miRNA alterations during progressive stages of EAC[55]. They found 26 miRNAs that are highly and frequently deregulated in BE and EAC when compared to paired normal esophageal squamous tissue[55]. They identified miR-31 and −375 as potential markers of progression during early and late stages of tumorigenesis, respectively. In an independent study, Wu et.al confirmed miR-375 as a miRNA being downregulated exclusively in cancers, additionally supporting its role as a marker of cancer progression in BE[21].

Although significant progress has been made in characterizing miRNA alterations in BE and EAC, 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 BE and EAC. 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. In addition, the differences between disease and normal states are often suboptimal for development of robust biomarkers. These limitations will need to be overcome for microRNA based biomarkers to be clinically useful.

## Clonal alterations and LOH (loss of heterozygosity)

Maley, Reid and colleagues have conducted numerous studies describing the relationship between clonal diversity and clonal expansions and the risk of BE progression. One prospective study of 268 BE patients evaluated whether clonal expansions during the progression of BE leads to homogenous cell populations or results in clonal diversity[34]. The authors found that patients with greater clonal diversity had greater risk of progression to EAC ($p<0.001$). In a follow-up study, this group compared clonal diversity in 79 BE 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[56]. Meanwhile, individuals that progressed to EAC 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 BE may need to incorporate assessment of structural genomic alterations and multiple foci of BE 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 EAC, 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 EAC within 2 years (14.2%), compared with patients with a non-polysomic FISH result (1.4%, $P < 0.001$)[32].

## 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 BE tissue indicates an increased risk for EAC, especially for BE with low grade dysplasia.

In the last 10 years, a series of studies by investigators at Erasmus MC University Medical Center found that increased TP53 expression in BE, determined by immunohistochemistry (IHC), preceded development of HGD/EAC by several years and that TP53 expression was an important risk factor for HGD/EAC with a hazard ratio (HR) of 6.5 (95% CI: 2.5–17.1)[57, 58] In this 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, BE length, and esophagitis (relative risk [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 BE patients in Ireland, TP53 protein overexpression did not predict progression in a multivariate analysis[30]

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% confidence interval, 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[59]. This assay is a promising tissue based prediction assay for progression to HGD or EAC 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 microRNAs, 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[65–68]. Most recently, in a proof of principle study Qin et 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 (EAC and esophageal squamous cell cancer) overall, and 43%, 64%, 77%, and 92% of stages I, II, III, and IV, respectively[69]. (See Table 2.)

## Conclusions

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

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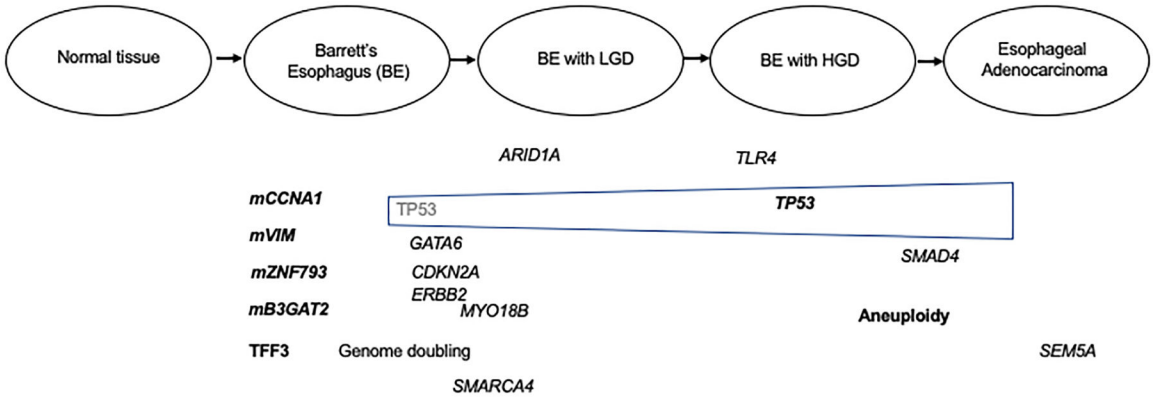


**Figure 1:**

Representation of the Barrett's esophagus to esophageal adenocarcinoma progression sequence and accompanying genomic and epigenomic alterations. The alterations show 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.

**Table 1:**

Validated BE early detection markers

| Biomarker | Method | Study Design | AUC | Sensitivity | Specificity | Ref. |
|---|---|---|---|---|---|---|
| TFF3 | IHC (cytosponge) | BEST-2: Case-control (N=1110) | | 80% | 92% | [42] |
| *mVIM* and *mCCNA1* | bsNSG (Esocheck device) | Case-control Validation cohort (N=86) | | 90% | 92% | [36] |
| *mB3GAT2* | methyLight PCR (endoscopic brushings) | Case-control Validation cohort (N=66) | 0.95 | 80% | 86% | [37] |
| *mZNF793* | methyLight PCR (endoscopic brushings) | Case-control Validation cohort (N=66) | 0.96 | 80% | 93% | [37] |
| *mTFPI2* | methyLight PCR (cytosponge) | Case-control Validation cohort (N=278) | 0.88 (0.84–0.91 | 82% | 96% | [41] |
| *mTWIST1* | methyLight PCR (cytosponge) | Case-control validation cohort (N=278) | 81% (0.77–0.86) | 70% | 93% | [41] |

IHC=immunohistochemistry, bsNSG=bisulfite next generation sequencing

Table 1 below summarizes BE biomarkers that have been evaluated in clinical cohorts.

**Table 2:**

Candidate BE risk stratification markers

| Biomarker | Study Design | Sample Size | Outcome |
|---|---|---|---|
| Abnormal DNA ploidy, 9pLOH, 17pLOH[34] | Prospective Cohort | N=243 | RR=38.7<br>(95% CI 10.8–138.5) |
| Aneuploidy, tetraploidy[60] | Retrospective analysis | N=322 | RR=11 (95% CI 5.5–21) |
| LOH by FISH: 17p13.1[61] | Retrospective analysis of surveillance cohort | N=151 | 5% of NDBE<br>9% of LGD<br>46% of HGD |
| CNA and LOH by FISH:<br>8q24, 9p21, 17q11.2, and 20q13.2[62] | Prospective | N=138 | LGD: sens 70%, spec 89%<br>HGD: sens 84%, spec 93%<br>EAC: sens 94%, spec 93% |
| Hypermethylation of CDKN2A, RUNX3, HPP1[63] | Retrospective and longitudinal | N=53 | CDKN2A OR 1.74<br>RUNX3 OR 1.8<br>HPP1 OR 1.77 |
| Jin methylated gene panel[64] | Retrospective, multi-center, double-blinded | N=50 progressors<br>N=145 non-progressors | AUC=0.843 at 2 years<br>AUC=0.829 at 4 years |
| Tissue Cypher[59] | Case-Control multi-center | N=145 non-processors, 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) |