CHAPTER 2

Predictive biomarkers for Barrett’s esophagus: so near and yet so far

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Biomarkers in premalignant conditions of the gastrointestinal tract

ABSTRACT

Barrett’s esophagus (BE) is the strongest risk factor for the development of esophageal adenocarcinoma (EAC). However, the risk of cancer progression is difficult to ascertain in individuals as a significant number of patients with BE do not necessarily progress to EAC. There are several issues with the current strategy of using dysplasia as a marker of disease progression. It is subject to sampling error during biopsy acquisition and interobserver variability among gastrointestinal pathologists. Ideal biomarkers with high sensitivity and specificity are needed to accurately detect high risk BE patients for early intervention and appropriate cost-effective surveillance. To date, there are no available molecular tests in routine clinical practice despite known genetic and epigenetic aberrations in the Barrett’s epithelium. In this review, we present potential biomarkers for the prediction of malignant progression in BE. These include markers of genomic instability, tumor suppressor loci abnormalities, epigenetic changes, proliferation markers, cell cycle predictors, and immunohistochemical markers. Further work in translating biomarkers for routine clinical use may eventually lead to accurate risk stratification.
Predictive biomarkers for Barrett’s esophagus: so near and yet so far

INTRODUCTION
Barrett’s esophagus (BE) refers to the replacement of normal squamous epithelium by metaplastic columnar epithelium in the distal esophagus. It is considered the most established risk factor for the development of esophageal adenocarcinoma (EAC). With increasing grades of dysplasia, the risk of progression to EAC also increases. The presence of BE-associated high grade dysplasia (HGD) carries the highest risk of EAC progression up to 10% annually. However, only approximately 0.2-0.5% of patients with non-dysplastic BE would develop EAC annually.

In spite of a low rate of progression from non-dysplastic BE to EAC, the incidence of EAC has increased significantly in western countries in the past four decades and even in some Asian regions where the disease was previously rare. The increasing incidence of invasive EAC with its dismal 5-year survival rate of less than 15% has intensified the interest in detecting dysplastic changes in BE. Successful management of BE dysplasia relies on accurate risk stratification which may facilitate cost-effective surveillance and early treatment to prevent progression to EAC.

Need for biomarkers
The current diagnosis of BE relies on the endoscopic recognition of salmon-colored mucosa above the gastroesophageal junction. The presence of dysplasia has been the current standard in assessing the risk of EAC progression. However, detection and grading of dysplasia are fraught with several limitations.

Reliable biomarkers are crucial in the pursuit of distinguishing BE patients who are predisposed to develop EAC. We previously reported our nationwide survey of U.S. gastroenterologists and their satisfaction with the current BE surveillance strategies. Only 50% of gastroenterologists were satisfied with the currently available strategies. Among the different factors identified, they were least satisfied with the inability of surveillance histology to predict the risk of progression among patients with non-dysplastic BE. More than 85% of survey participants would be willing to use fluorescence in situ hybridization (FISH)-based testing for the detection of gene copy abnormalities if proven to be effective in predicting risk of progression. Gastroenterologist were willing to extend the surveillance interval on patients with non-dysplastic BE and low grade dysplasia (LGD) if a FISH-based test can stratify high risk individuals accurately.

Even with the use of enhanced endoscopic imaging techniques such as narrow band imaging, confocal laser endomicroscopy, and chromoendoscopy, the current state of endoscopic detection and biopsy acquisition are subject to sampling error. The Seattle protocol which recommends targeted biopsy of suspected lesions and four quadrant biopsies every 1 to 2 cm in the entire BE segment is time-consuming, arduous, and subject to sampling bias. These factors have been reported to contribute to the poor adherence among community-based gastroenterologists. Even with accurate biopsy acquisition, histopathologic interpretation is open to wide inter-observer variability among pathologists.
There are several proposed biomarkers of BE in the literature. We conducted a systematic literature search in PubMed and Scopus using the search terms: “Barrett’s esophagus”, “biomarkers” and “biological marker” from 1980 to 2011. We found 1069 citations on the topic of biomarkers in BE (Figure 1). The increasing publications through the years reflect the on-going search for effective biomarkers as well as the lack of a clinically-validated prognostic tool in BE.

**Candidate biomarkers**

Our understanding of cancer pathogenesis in BE has progressed with advances in molecular biology. Using the techniques of DNA microarray, epigenetics, and proteomics, various biomarkers that contribute to the progression from BE to EAC have been detected in preclinical studies. Some of them have exciting potential as biomarkers in risk stratifying BE patients with higher risk for progression and even prediction of response to therapy. However, very few are ready for clinical use. Their implementation is still hindered by differences in reproducibility of results, inadequate sample size, and need for multi-center prospective studies.13, 14

According to the recommendations for biomarker development proposed by the Early Detection Research Network (EDRN), five phases of study are required to approve a biomarker for clinical use.15 After an exploratory phase in which potential markers are identified (phase 1), a clinical assay is developed (phase 2). Subsequently, these markers need to be validated in
retrospective (phase 3) and prospective (phase 4) studies, respectively. Most of the current biomarkers have been evaluated in phase 3 and few in phase 4 studies, but none have been validated in a phase 5 study. Phase 5 or cancer control studies are designed to evaluate the impact of a biomarker test on the population disease burden and primary outcomes include costs and mortality rates. In this review, we present biomarkers that have the potential for clinical application. These predictive biomarkers and panels are presented below and summarized in Table 1.

**Genomic instability**

The similarity of genetic patterns between BE and EAC demonstrated by sequence-verified human cDNA microarray, support the hypothesis that BE is an intermediate step towards EAC, and the common changes at the molecular level form the foundation for carcinogenesis. Genomic instability has been found to be a poor prognostic marker in BE and is reflected by chromosomal alterations, deletions, point mutations, methylation abnormalities and loss of heterozygosity (LOH). Some of these changes are considered early events and insufficient for the development of cancer, but other key molecular changes implicated in gatekeeper events also have the potential to serve as biomarkers for risk stratification.

**DNA content abnormalities**

DNA content abnormalities refer to the numerical and/or structural changes in chromosomes including aneuploidy (i.e. a cell containing an abnormal number of chromosomes) and tetraploidy (i.e. the state of having four complete sets of chromosomes instead of two). Aneuploidy and tetraploidy as assessed by flow cytometry can be used as a biomarker with a significant predictive value, especially in subgroups of patients with non-dysplastic BE or LGD. In a retrospective analysis of a prospectively maintained database of 322 BE patients, the presence of aneuploidy and/or tetraploidy had a relative risk (RR) of 11 (95% CI = 5.5 - 21) for neoplastic progression, compared with those without baseline abnormalities. LOH represents the loss of normal function of one allele of a gene in which the other allele was already inactivated. A chromosomal instability panel combining aneuploidy and tetraploidy with 9p LOH and 17p LOH, was a strong predictor of EAC (RR 38.7, 95% CI: 10.8-138.5) in a long-term follow-up study of 243 BE patients. Patients without baseline abnormalities had a 12% cumulative EAC incidence in 10 years, whereas patients with 9p LOH, 17p LOH and DNA content abnormalities had a 79% EAC incidence.

Changes in glycan expression and specific glycan-binding proteins (lectins), such as Wheat Germ Agglutinin and Aspergillus oryzae lectin (AOL) have been shown to arise in the development of EAC. In a recent case-control study with 89 patients with progression and 291 without progression, several established and novel biomarkers were investigated. A panel compromising LGD, abnormal DNA ploidy and AOL most accurately identified patients with progression as compared to those without progression, with relatively simple techniques...
including image cytometry and histochemistry that can be applied on paraffin-embedded tissue samples.\textsuperscript{23} Despite good results of ploidy-status as a predictive marker, its use in clinical practice is still limited.

**Tumor suppressor loci abnormalities**

LOH for p53 has been demonstrated to be an effective biomarker to predict risk of progression from LGD to HGD and EAC. In a phase 4 study of 256 patients with baseline evaluation, Reid et al. reported that p53 LOH was associated with a 16-fold increase in the risk of progression to cancer (p<0.0001).\textsuperscript{22} Mutations of the p53 gene led to the synthesis of a P53 protein with a longer half-life resulting in accumulation of the protein which enables immunohistochemical detection in biopsy specimens. Detection of P53 overexpression by immunohistochemistry (IHC) could be used in addition to dysplasia more easily than LOH analysis which requires genotyping. Although P53 overexpression increased the risk of cancer by almost 12 fold, it may have limited value as a prognostic marker in patients with non-dysplastic BE as only 32.4\% of patients with progression showed overexpression of P53 in their initial biopsy.\textsuperscript{23}

P16 plays a crucial role in cell cycle control and the alternation of p16 is frequently observed in up to 85\% of esophageal adenocarcinomas.\textsuperscript{24} Hypermethylation of p16, assessed by real-time quantitative methylation specific PCR in a retrospective study of 53 cases, was independently associated with an increased risk of progression from IM to HGD or EAC (OR 1.74; 95\% CI 1.33–2.20).\textsuperscript{25} Furthermore, allelic loss of p16, detected by FISH can help predict lack of response to photodynamic therapy in patients with BE associated HGD and intramucosal cancer.\textsuperscript{26} Loss of p16 is considered an early event in the development of dysplasia.\textsuperscript{27} The fact that there is also significant prevalence of p16 loss in non-dysplastic BE questions the precise utility of this biomarker for assessing risk of neoplastic progression.\textsuperscript{28} Maley et al. proposed a linear chain evolution model of carcinogenesis in BE, in which p16 variations were regarded as the initial mutation which resulted in clonal expansion that would sweep across the entire BE.\textsuperscript{29} This would create a field where subsequent genetic variations such as p53 LOH and new clonal expansions could arise. Eventually, the accumulation of mutations may lead to a cancerous clone. However, this hypothesis has been challenged by detection of independent multiple genetically distinct clones present at the crypt level.\textsuperscript{30} In conclusion, LOH of p53 is a likely biomarker to predict progression. Its efficacy and validity need to be confirmed in large scale, population-based studies.

**Fluorescence in situ Hybridization**

FISH is a technique in which small fluorescently labelled DNA probes are used for detection of chromosomal and specific gene aberrations (Figure 2). The assessment of ploidy status in BE by FISH with the use of centromeric probes of chromosome 7 and/or chromosome 17 was more sensitive than DNA cytometry to detect chromosomal abnormalities in BE. Gains of chromosome 7 and/or 17 were detected in 13\% of non-dysplastic cases, increased with dysplasia stage and detected HGD/EAC with a sensitivity and specificity of 85\% and 84\%,
respectively. FISH analysis with a probe set consisting of gene locus 8q24 (C-MYC), 9p21 (p16), 17q12 (HER2), and 20q13, was able to detect LGD, HGD and EAC with a sensitivity of 50%, 82% and 100%, respectively. Recently, preliminary data from a long-term prospective follow up study have shown promising results in identifying high risk BE patients with a novel FISH assay including the tumor suppressor genes p53, p16 and centromeric probes of chromosome 7 and 17 to detect aneuploidy. Having a positive result correlated with a hazards ratio of 5.5 (p= 0.002) for the progression of both IM to LGD and from LGD to HGD.

Clonal diversity

Majority of recent research in BE dysplasia has focused on chromosomal instability within the BE tissue. Few studies have addressed clonal evolutionary mechanisms that drive neoplastic progression. Clonal diversity refers to the coexistence of multiple distinct clones derived from a number of genetic instabilities. Merlo et al. measured clonal diversity in a cohort of 239 BE patients. All diversity measures, including DNA content, LOH, microsatellite shifts, sequence mutations in p53 and p16 were strong predictors of progression (p<0.001). However, the use of clonal diversity as a predictive marker is limited by its laborious and complicated methodology.

Figure 2. Fluorescence In Situ Hybridization with representative cytology samples. Representative sample of brush cytology specimen after FISH with probes for 8q24 [MYC] [aqua], 9p21 [p16] [red], 17q11.2 [ERBB2] [green], and 20q13.2 [ZNF217] [gold]. The normal cell has 2 signals from each of the 4 probes. The abnormal cell was found to have gain of multiple probes.
Epigenetic changes and methylation

Epigenetics is defined as heritable changes in gene function that occur without a change in DNA sequence. Methylation of CpG islands in promoter genes is the most important epigenetic change in human cancer pathogenesis and is associated with silencing of many tumor suppressor genes. Recently, several methylation biomarkers have been assessed to predict the progression from dysplasia to carcinoma. In a retrospective multicenter study, a methylation biomarker panel which combined eight genes (\textit{p16}, \textit{RUNX3}, \textit{HPP1}, \textit{NELL1}, \textit{TAC1}, \textit{SST}, \textit{AKAP12}, and \textit{CDH13}), was evaluated in 195 BE patients. Sensitivities of progression prediction approached 50%. Similar to the determination of clonal diversity, this approach requires expertise that is not readily available in routine laboratory use. The value of \textit{p16} methylation as a single biomarker has been discussed in the tumor suppressor section.

Proliferation markers

There is controversy whether abnormal cellular proliferation is associated with higher grades of dysplasia in BE. The differences in techniques, proliferative indices, and histological architectures between columnar and squamous epithelium might contribute to these discrepancies. In a retrospective case control study, overexpression of the proliferation maker Mcm2 on surface cells of biopsy specimens was associated with progression to EAC. Mcm2 was expressed in 28.4% of patients with progression and 3.4% without progression (\textit{p}<0.0001). Ki67 is a nuclear protein that is associated with cellular proliferation. In a cohort study of 362 BE patients with mean follow-up of 6.3 years, increased S and G2 cell cycle fractions at baseline biopsy were significantly associated with cancer progression, but Ki67-positive proliferative fractions were regarded as adaptive changes to reflux and not associated with risk of progression (\textit{p} = 0.03 and \textit{p}<0.0001, respectively).

Cell cycle predictors

Most cancer cells have dysregulated cell cycle checkpoints resulting in the accumulation of genetic aberrations. Overexpression of cell cycle-related proteins, such as cyclin D1, has been detected in several studies and shown to be likely implicated in the process of cancer progression in BE. In a case-control study, overexpression of cyclin D1 was associated with an increased risk of progression to EAC (OR 6.85; 95% CI 1.57-29.91). However, these findings were not replicated in a larger population-based case-control study. In another case-control study, surface expression of cyclin A has been shown to increase the risk of cancer progression (OR 7.5; 95% CI 1.8-30.7). Further research efforts are needed to confirm the predictive values of cyclins.

Mitochondrial DNA

Alterations in mitochondrial DNA (mtDNA), a small circular genome located in the mitochondria, have been implicated in the multistep process of carcinogenesis. In patients
with non-dysplastic BE, 53% exhibited mtDNA mutations in their Barrett’s specimen but not in adjacent normal tissue. Deletion of 4977bp, one of the most widespread deletions of the mitochondrial genome, was found in 15.4% in IM; 40% in LGD; 69.2% in HGD and 90% in paratumoral tissue in 70 specimen of patients with BE. Of interest, the frequency of the deletion was only present in 16.7% of EAC specimens. Therefore, the increased frequency of mtDNA changes may correlate with the evolution from IM to dysplasia.

**Telomere shortening**

Telomeres protect the end of chromosomes from degradation, fusion, and rearrangements during DNA replication and shorten with each cell division. Telomere shortening may cause chromosomal instability and has gained attention in the research of aging and cancer. Based on epidemiological studies, risk factors for BE such as gastroesophageal reflux, cigarette smoking, and central obesity may reduce telomere length. Leukocyte telomere shortening, measured by quantitative PCR in blood samples in a cohort of 300 BE patients, was significantly associated with increased EAC risk (HR 4.18; 95% CI 1.60-10.94) after adjusting for other risk factors of EAC. The mechanisms that explain the association between telomere shortening in leukocytes and cancer progression in BE patients is unknown. However, it is hypothesized that telomere shortening in these patients happens as a consequence of inflammation and oxidative stress associated with BE. Despite the fact that telomere length is vulnerable to various other oxidative damages, these results suggest that leukocyte telomere shortening may have potential as a biomarker in a cancer risk model.

**Selenoprotein P**

Clinical trials have suggested a protective effect of selenium supplementation on the risk of EAC. A recent phase 4 study could not confirm an association between serum selenium levels and risk of progression, but selenoprotein P (SEPP1), a carrier of selenocysteins with antioxidant properties, was associated with an increased risk of developing EAC in patients with BE (HR 3.95; 95% CI 1.42–10.97). Further research is warranted to clarify the underlying mechanism of this finding.

**Other biomarkers**

In addition to the biomarkers mentioned above, several other potential biomarkers for the prediction of neoplastic progression in BE have been studied. Prior studies have explored tumor cell markers such as HER2/neu, APC, MYC, SMAD4, EGFR and 20q13 in cross sectional analyses. Overexpression of these markers was associated with dysplastic progression. Future studies are still needed to confirm their utility beyond phase 3 studies.
Table 1. Summary of Molecular Biomarkers Predicting Progression in Patients with Barrett’s Esophagus

<table>
<thead>
<tr>
<th>Biomarker panels</th>
<th>Phase</th>
<th>Sample size</th>
<th>Baseline histology</th>
<th>Technique</th>
<th>End point</th>
<th>Predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-gene methylation panel†</td>
<td>3</td>
<td>195</td>
<td>IM</td>
<td>RT-PCR</td>
<td>HGD or EAC</td>
<td>Predicted 50% of progressors 37</td>
</tr>
<tr>
<td>DNA content abnormalities</td>
<td>4</td>
<td>243</td>
<td>IM</td>
<td>Sequencing, LOH, MS-PCR, flow cytometry</td>
<td>EAC</td>
<td>RR 38.7 (95% CI: 10.8-138.5) 20</td>
</tr>
<tr>
<td>Expert LGD, aneuploidy,</td>
<td>3</td>
<td>380</td>
<td>IM, ID or LGD</td>
<td>Histochemistry, IHC, image cytometry, DNA analysis</td>
<td>EAC</td>
<td>BE with baseline LGD: OR 3.90 (95% CI 2.39-6.37) 20</td>
</tr>
<tr>
<td>Aspergillus oryzae lectin§</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BE without LGD OR 3.31 (95% CI 1.81-6.05) 20</td>
</tr>
<tr>
<td>DNA content abnormalities</td>
<td>3</td>
<td>322</td>
<td>IM, ID or LGD</td>
<td>Flow cytometry</td>
<td>EAC</td>
<td>RR 11 (95% CI: 5.5-21) 3</td>
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<tr>
<td>Aneuploidy/tetraploidy</td>
<td>4</td>
<td>256</td>
<td>IM, ID or LGD</td>
<td>Locus specific PCR</td>
<td>EAC</td>
<td>OR 16 (95% CI: 6.2-39) 22</td>
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<tr>
<td>Tumor suppressor loci</td>
<td>4</td>
<td>48</td>
<td>IM, ID or LGD</td>
<td>IHC</td>
<td>HGD or EAC</td>
<td>RR 5.7 34</td>
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<tr>
<td>P53 LOH</td>
<td>3</td>
<td>97</td>
<td>IM</td>
<td>IHC</td>
<td>HGD or EAC</td>
<td>OR 11.7 (95% CI: 1.93-71.4) 23</td>
</tr>
<tr>
<td>P53 staining</td>
<td>4</td>
<td>48</td>
<td>IM, ID or LGD</td>
<td>RT-PCR</td>
<td>HGD or EAC</td>
<td>OR 1.74 (95% CI: 1.33-2.20) 25</td>
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<tr>
<td>Epigenetics</td>
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<tr>
<td>P16 methylation</td>
<td>3</td>
<td>53</td>
<td>IM/LGD</td>
<td>RT-PCR</td>
<td>HGD or EAC</td>
<td>OR 0.81 (95% CI: 0.14-4.5) 23</td>
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<tr>
<td>Proliferation</td>
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<tr>
<td>Mcm2</td>
<td>3</td>
<td>27</td>
<td>IM</td>
<td>IHC</td>
<td>EAC</td>
<td>OR 7.5 (95% CI: 1.8-30.7) 46</td>
</tr>
<tr>
<td>Clonal diversity</td>
<td>4</td>
<td>239</td>
<td>IM</td>
<td>Various</td>
<td>EAC</td>
<td>Significant predictors of progression (p&lt;0.0001) in a Cox proportional hazards model. 34</td>
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<tr>
<td>Clonal diversity measures†</td>
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<tr>
<td>Cell cycle markers</td>
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<tr>
<td>Cyclin A</td>
<td>3</td>
<td>48</td>
<td>IM</td>
<td>IHC</td>
<td>HGD or EAC</td>
<td>OR 6.85 (95% CI: 1.57-29.91) 44</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>3</td>
<td>307</td>
<td>IM</td>
<td>IHC</td>
<td>EAC</td>
<td>OR 0.81 (95% CI: 0.14-4.5) 23</td>
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<tr>
<td>Serum biomarkers</td>
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<tr>
<td>Leukocyte telomere length</td>
<td>4</td>
<td>300</td>
<td>Variable</td>
<td>TQ-PCR</td>
<td>EAC</td>
<td>HR 4.18 (95% CI: 1.60-10.94) 50</td>
</tr>
<tr>
<td>Selenoprotein P</td>
<td>4</td>
<td>361</td>
<td>Variable</td>
<td>ELISA</td>
<td>EAC</td>
<td>HR 3.95 (95% CI: 1.42-10.97) 52</td>
</tr>
</tbody>
</table>

Abbreviations: IM, intestinal metaplasia; LGD, low grade dysplasia; ID, indefinite for dysplasia; HGD, high grade dysplasia; EAC, esophageal adenocarcinoma; LOH, loss of heterozygosity; IHC, immunohistochemistry; MS-PCR, methylation-specific PCR; TQ-PCR, telomere quantitativePCR; OR, odds ratio; RR, risk ratio.

†Methylation panel included p16, RUNX3, HPP1, NELL1, TAC1, SST, AKAP12, and CDH13.

‡ Panel included aneuploidy, tetraploidy, LOH of 9p and 17p.

§ Odds ratio per point increase in a risk stratification model using Expert LGD, aneuploidy and Aspergillus oryzae lectin.

¶ Clonal diversity measures included DNA content, LOH, microsatellite shifts, sequence mutations in p53 and p16.

22
Predictive biomarkers for Barrett’s esophagus: so near and yet so far

**Conclusion**

This review summarizes the characteristics and potential use of biomarkers in BE. Most biomarkers have been evaluated in EDRN phase 3 and few in phase 4 studies but none has been validated in a phase 5 study. The ability to detect and predict BE patients who will progress to EAC remains to be an elusive target. Conducting phase 4 and 5 studies may be a major challenge considering the requirements of a large sample size and long follow-up period due to the low incidence of EAC developing from non-dysplastic BE. An ideal biomarker should be cost-effective, non-invasive, easily administered, and with better diagnostic performance as compared to dysplasia detection in biopsies. Because a single biomarker is often inadequate for intended clinical application, a panel of molecular biomarkers and clinical factors might be needed for better sensitivity and specificity.

At this time, validated biomarkers that can be performed on a routine clinical basis are not yet available. Despite the previously mentioned limitations, it is reasonable to believe that further work on translating biomarkers into routine clinical use may eventually lead to improved surveillance strategies for BE dysplasia and early EAC.

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Predictive biomarkers for Barrett’s esophagus: so near and yet so far


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26


