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Early Events During Neoplastic Progression in Barrett's Esophagus

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SUMMARY

Advances in genomic analysis and sequencing, transcriptomics and proteomics are rapidly increasing our understanding of the complexity, redundancies and heterogeneity among cancers. Challenges to risk stratification, prevention and early detection will become great if, as expected, EA has similar complexity compared to other cancers. Discovery of very large possible combinations of biomarkers for BE risk assessment for progression to EA may overwhelm the translational research process for biomarker validation. One approach for risk stratification and early detection would be to search for fundamental biomarkers of progression, such as mutation rate, generation of diversity and clonal expansions. However, our previous studies and others have reported that low density STR biomarkers combined with DNA content flow cytometry could stratify patients into clinically relevant risk groups to manage the cancer risk for five years into the future reasonably well.¹¹⁷ With new SNP based technology and larger cohort studies with greater sample sizes, there is promise to achieve better biomarkers for EA risk stratification and early detection for clinical use because genome-wide measures of chromosome instability and 17pLOH have shown promise in all prospective studies. Such a platform could be readily adapted to risk stratification and evolutionary biomarkers of progression, including measures of clonal diversity and expansions.

INTRODUCTION

Age adjusted cancer mortality has remained relatively constant over the last 50 years in spite of intense efforts to cure advanced malignancies by advances in surgery, chemotherapy, radiation therapy and combined modality therapy¹ (ACS Cancer Statistics 2009). This has lead to efforts to reduce cancer mortality by early detection and prevention. Ironically, these efforts have lead to the surprising discovery that many conditions affecting multiple organs that are morphologically classified as “premalignant” or even “malignant” follow indolent courses with many or even most patients dying of unrelated causes. The paradox of early detection is overdiagnosis of early indolent conditions and underdiagnosis of life threatening neoplasms. As a result, there has been increasing recognition of the importance of biomarkers for risk assessment to guide cancer prevention and early detection efforts. The challenge for personalized medicine is accurate risk assessment so that cancer prevention

and early detection can focus on reducing mortality in those patients most likely to die of cancer while reassuring low-risk patients of their low risk.

Although times have changed since Norman Barrett revisited the condition named for him², the lessons he learned remain applicable today. The definition of Barrett's esophagus (BE) has changed several times, and there is no universally accepted definition even today.^{3, 4} It has been defined as an intestinal metaplasia and a neoplasm.⁵⁻⁷ BE has been called a complication of chronic symptomatic gastroesophageal reflux disease (GERD), yet some features of BE appear instead to be a successful adaptation to the harsh intra-esophageal environment of GERD.⁸ BE has also frequently been called a premalignant condition, yet most persons with BE die of causes other than esophageal adenocarcinoma (EA).⁹⁻¹³ The challenges facing the management of BE are the same as those in many other organs, including prostate, lung, breast, thyroid, kidney and others, where morphologic "pre-malignant" or "malignant" diagnoses follow an indolent course. However, BE is easier to study because periodic endoscopic biopsy surveillance is a standard of care⁵ in contrast to others, which are removed when detected, such as a colonic adenoma, or which cannot be systematically sampled because of the potential for adverse outcomes of tissue sampling. BE is a model for understanding those factors that determine whether these conditions will remain stable throughout life or progress to a life-threatening malignancy.

POPULATION STUDIES BARRETT'S ESOPHAGUS (BE) AND ESOPHAGEAL ADENOCARCINOMA (EA)

The incidence of EA has been increasing more rapidly than any other cancer in the US and much of the western world for the past three decades.^{14, 15} EA is a highly lethal cancer with mortality greater than 85% unless detected early.¹⁶ BE is the only known precursor of EA, but the rate of progression from BE to EA is only about 6 to 7 per 1,000 person-years^{17, 18}, and 90-95% of persons with BE die of causes unrelated to EA.⁹⁻¹³ Population-based case control and cohort studies have identified many risk and protective associations for EA. Population attributable risk is most closely associated with four factors, including symptomatic GERD, obesity, diet and tobacco use.¹⁹ In part, this may reflect the large population of persons with GERD and a similarly large population affected by obesity and a prior history of tobacco use. For example, Gallup polls report that 44% of the adult population of the United States have symptomatic GERD, yet, only about 8,000 people develop EA annually.¹⁵ Even though EA is strongly associated with symptomatic GERD, many people can develop EA or BE without frequent reflux symptoms. For example, nearly 50% of patients with EA report an infrequent history of GERD symptoms in population-based studies.^{20, 21} In addition, two recent studies from Sweden and Italy reported that the prevalence of BE is nearly as great in those without reflux symptoms as in those with symptomatic GERD.^{22, 23} In the absence of evidence that screening reduces mortality of EA or identification of a patient subset at sufficiently high risk to warrant screening, earlier recommendations of the American College of Gastroenterology Guidelines to screen patients with chronic GERD symptoms for BE have recently been withdrawn and the guidelines now state "screening for Barrett's esophagus in the general population cannot be recommended at this time." Similarly, a recent American Gastroenterological Association

Institute technical review on the management of gastroesophageal reflux disease concluded that "...no direct evidence supports the use of endoscopy as a screening test for Barrett's esophagus or esophageal adenocarcinoma in the setting of chronic GERD."²⁴ Two decades of screening patients with GERD for BE have impacted neither the incidence nor mortality of EA, but they have detected a very large population of persons with an indolent condition who are exposed to expensive and invasive procedures, which carry their own quantifiable risks, from which the average patient can anticipate little benefit. Here, we will examine the early biology of BE to guide efforts to improve detection of patients whose high risk of progression to EA warrants intervention and/or early detection and to reassure those at low risk so that they may avoid expensive, invasive and potentially harmful procedures.

The current strategy for identification of biomarkers for risk stratification and early detection in BE is largely based on discoveries using genomic, transcriptomic, and proteomic technologies that have rapidly advanced our understanding of the complexity of cancer.²⁵⁻³⁴ Recent studies reporting DNA sequences of several cancer genomes, including colon, breast, pancreas and glioblastoma, have revealed many more mutations than were expected, affecting multiple steps in multiple biological pathway.³⁵⁻³⁷ Based on the sequencing data, it has recently been suggested that strategies to cure cancers by targeted therapies may have to focus on controlling multiple pathways rather than individual genes.³⁸

An alternative approach is to identify persons at high risk for progression to cancer for cancer prevention and early detection strategies. Although the EA genome has not yet been sequenced, large scale datasets from genomic, expression and proteomic studies have greatly expanded the number of candidate biomarkers in BE and EA.²⁶⁻³⁴ If similar numbers of (epi)genetic alterations and pathways exist in BE, then the challenges for risk stratification, cancer prevention and early detection could increase substantially, especially if the EA cancer genome, as would be expected, also has mutations in multiple genes in multiple biological pathways. The challenge to cancer prevention and early detection may be similar, but it may be possible to develop measures of overall genome instability that distinguish high-risk patients from those at low risk for risk stratification of BE and early detection or prevention of EA. A longitudinal study that follows a cohort of patients with BE to identify those who do and do not progress to EA is the "gold standard" to distinguish indolent alternations in the BE mucosa from truly premalignant abnormalities that will progress to EA. This chapter will focus on what can be learned from this type of study.

CURRENT MANAGEMENT OF THE RISK OF ESOPHAGEAL ADENOCARCINOMA IN BARRETT'S ESOPHAGUS

Periodic endoscopic biopsy surveillance for early detection is a current standard of care for managing the cancer risk in BE.⁵ Biopsies are graded for dysplasia as negative, indefinite, low-grade dysplasia (LGD), high-grade dysplasia (HGD) and EA.^{39, 40} There are limitations to this approach for managing the cancer risk in BE. Dysplasia classification is subjective, and there is substantial observer variation in diagnosis even among expert GI pathologists.^{39, 40} This variation is increased with community pathologists,⁴¹ and it is common practice to send slides with suspected dysplasia for second opinions from expert GI pathologists, driving up the overall cost of medical care and increasing patient anxiety.

The goal of the original dysplasia classification system was to develop a standard for stratification of the risk of progression from BE to EA.³⁹ Another limitation of dysplasia classification is that the studies that have been reported cast some doubt on the robustness of dysplasia for risk stratification. For example, HGD appears to confer an increased risk for progression from BE to EA although the magnitude of the risk varies considerably in different centers. Studies from referral centers have reported high rates of progression with five-year cumulative EA incidences greater than 50%, but these high rates of progression may reflect selection in the referral process.⁴²⁻⁴⁴ When selection is eliminated, reported five-year cumulative incidences of EA in HGD range from 8% to about 30%.^{42, 45} Progression studies for dysplasia diagnoses less than HGD, as well as molecular progression studies, have been in general confounded by use of HGD as a surrogate endpoint. Since most patients with HGD do not progress to EA over relatively long follow-up intervals, HGD does not meet accepted standards as a surrogate endpoint because it does not accurately reflect the incidence of EA or EA mortality as valid endpoints.^{46, 47} In general, variation in observer diagnosis has not been considered in studies that report progression from LGD to HGD, and misclassification may contribute to what is called “progression.”⁴⁸ In studies using EA as an endpoint, LGD appears to have a low rate of progression.^{42, 45, 49, 50} In many studies, LGD appears to be transient with many more patients reversing to lesser grades of dysplasia than advancing to EA.⁴⁹⁻⁵¹ Two longitudinal studies have suggested that quantifying features of dysplasia may improve diagnostic accuracy for future progression to EA.^{52, 53} A further limitation of dysplasia classification is that it can occupy small and patchy regions of the BE segment.⁵⁴⁻⁵⁶ As a result, systematic biopsy protocols are recommended with 4 quadrant biopsies every one to two cm in the Barrett’s segment.⁵ Finally, a diagnosis of dysplasia does not lead to low-cost, non-invasive interventions to prevent progression to EA. For example, esophagectomy was routinely performed for a diagnosis of HGD in BE for nearly two decades until examination of the Medicare database reported that the mortality of esophagectomy ranged from 8.1% in high-volume hospitals to 23.1% in low-volume hospitals.⁵⁷ This was consistent with other studies using different databases.⁵⁸⁻⁶⁰ A randomized trial of aspirin and proton pump inhibitor therapy for BE is currently underway in the UK, but the results will not be available for some time.⁶¹ Ablation for a subset of patients proven to be at high-risk of progression to EA is another possible strategy, although no form of ablation has been reported to reduce EA mortality in a randomized trial. A randomized trial of photodynamic therapy for HGD reported decreased incidence of EA, but a non-significant increase in advanced stage (T2, T3) EAs in the PDT arm.⁶² Patients who developed advanced EAs were excluded from further analysis as “treatment failures” and cancer mortality may have been underestimated in the trial.⁶³ A preliminary report from a small randomized trial of radiofrequency ablation in BE had only one EA endpoint, making the results largely dependent on surrogate endpoints and difficult to interpret.⁶⁴ The long term effects of such treatment remain to be evaluated in adequately powered randomized control trials with valid EA and mortality endpoints.

BIOLOGY OF EARLY BARRETT’S ESOPHAGUS

Even though the definition of BE has changed many times over the years, this review will proceed from the literature in which BE has been called both a specialized intestinal

metaplasia and a neoplasm because it has properties of both. Although most research has concentrated on malignant risk of BE, a growing body of evidence has been building that indicates it has properties that may be beneficial to the host in the harsh environment of gastroesophageal reflux. BE itself does not cause symptoms; the symptoms are a consequence of reflux. Comparative studies have reported that persons with BE have less prevalent reflux symptoms than those with GERD alone.^{65, 66}

In contrast to the stratified squamous epithelium of the normal esophagus, BE is a columnar epithelium organized into crypts similar to those of the small and large intestines, hence the definition of “intestinal metaplasia” (Figure 1). It has long been known that BE secretes mucus that has been characterized by a variety of techniques,⁶⁷ and that the intracellular pathways that promote mucus secretion are disrupted in patients whose biopsies have aneuploidy or high-grade dysplasia.^{68, 69} In a recent comparative study, BE was reported to have a thick, adherent mucous gel layer (median 90 μ m; range 20-210) that was not present on normal human, pig or rat esophagi.⁷⁰ Another study reported that BE was an anion secreting epithelium with more than five-fold increased bicarbonate secretory capacity than esophageal squamous epithelium.⁷¹ In 2007, another study reported that BE had Claudin 18-rich tight junctions compared with esophageal squamous epithelium that could also contribute to improved resistance to acid reflux.⁷² An integrated genomics study reported that BE over-expresses genes involved in defense and repair processes.³⁰ Although BE stem cells have not yet been isolated, they are believed to reside at the base of the crypts by analogy with small intestine and colon. They give rise to transient amplifying cells that then differentiate and are sloughed into the lumen and excreted from the body. Thus, the architecture of BE places the stem cells at the base of the crypts where they are protected against damage by a number of mucosal defenses. Taken together, a broad, emerging range of evidence suggests that BE can be viewed as a successful adaptation to the harsh environment of chronic gastroesophageal reflux and that it generally follows an indolent, possibly beneficial, course.^{8, 30, 70-73}

NEOPLASTIC PROGRESSION IN BARRETT’S ESOPHAGUS

Genetic progression models were proposed for many neoplasms in the 1990s, and it is instructive to review them here. The most common approach was to compare frequencies of specific genetic abnormalities in neoplasms from different individuals representing different stages of neoplastic progression.⁷⁴ The colonic adenoma to carcinoma became the most famous of this type of genetic progression model although many others were published. However, the difficulty of this approach was illustrated by a study of colon cancers that reported only 6.6% of cancers arose through the hypothesized progression pathway.⁷⁵ It appeared that many pathways to cancer were identified among the different patients, and this has been confirmed by sequencing the genomes of several cancers.³⁵⁻³⁷ A large body of cross-sectional studies involving a wide variety of candidate biomarkers have been reported in BE, using grade of dysplasia to categorize the patients. However, normalization to dysplasia classification deeply embeds the limitations of dysplasia classification into interpretation of the biomarkers. For example, results may not be replicated in different centers simply because of observer variation in dysplasia diagnosis, even if the biomarkers themselves are robust. Although the reasons are multifactorial, few biomarkers reported in

cross-sectional studies have been validated in longitudinal studies as predictors of progression to EA. However, studies of neoplastic progression in individual patients using both space and time scales have shown promise in guiding effective biomarker discovery and validation.

SPACE AND TIME SCALES TO GUIDE DISCOVERY AND VALIDATION OF BIOMARKERS FOR RISK STRATIFICATION

CHROMOSOME INSTABILITY

Copy gain, loss and loss of heterozygosity (LOH)—A large body of evidence accumulated over more than two decades implicates chromosome instability in neoplastic progression from BE to EA. Evidence for genome-wide instability was first reported in the 1980s using DNA content flow cytometry, where a small fraction of BE samples and a large fraction of EAs were reported to contain aneuploid cell populations.⁷⁶⁻⁸⁴ Evidence for chromosome instability has continued to accumulate over the subsequent 20 years using cytogenetics,^{85, 86} loss of heterozygosity (LOH) analysis,⁸⁷⁻⁸⁹ fluorescent in-situ hybridization (FISH),⁹⁰⁻⁹⁶ comparative genomic hybridization (CGH),⁹⁷⁻¹⁰⁰ array CGH,^{27, 33} and SNP arrays.^{32, 101-103} Thus, chromosome instability has been confirmed in BE and EA by a large number of different laboratories using a variety of methods. Measures of chromosome instability also have the advantage that they have been evaluated over both spatial and time scales in BE, as described below.

Spatial scale: An alternative approach to determine relationships among genomic abnormalities during progression to cancer is to assess the order in which two events occur in the same neoplasm in the same patient, and then evaluate the frequency of this order across patients.¹⁰⁴⁻¹⁰⁶ This type of analysis is most easily performed in cases where clones expand across an epithelial surface, such as Barrett's esophagus, head and neck cancer,^{107, 108} bladder cancer,¹⁰⁹ lung cancer,¹¹⁰ and others. In BE, spatial relationships among clonal genetic abnormalities have been evaluated at the scales of individual crypts, biopsies and the BE segment.^{105, 106, 111, 112} Spatial data at the level of biopsies and the BE segment led to the hypothesis that CDKN2A abnormalities (LOH, methylation, mutation) were early events in BE that preceded TP53 abnormalities (LOH, mutation), and then DNA content tetraploidy and aneuploidy.^{105, 113-115} These small studies became the basis for larger, prospective studies of biomarkers and biomarker panels.^{116, 117} Systematic spatial sampling of the mucosal surface of cases that had progressed to EA also revealed large scale genomic heterogeneity with multiple different aneuploid cell populations that formed the foundation for future evolutionary analyses of diversity.^{105, 118, 119}

Temporal scale: Because periodic endoscopic biopsy surveillance is the current standard of care for early detection, it is possible to test hypotheses derived from spatial data in longitudinal studies to determine whether or not the candidate biomarkers might also be useful for risk stratification and/or early detection for EA. Some of the hypotheses generated from the spatial data in BE have been examined in longitudinal studies. Flow cytometric DNA content abnormalities were the first to be investigated for risk stratification in BE. In 1998, Teodori et al. reported a 13 year study of 362 samples from a cohort of 30 dysplasia

free individuals with BE.¹²⁰ None of 17 patients whose samples were all diploid progressed to EA (0%) compared with 3/13 whose biopsies had aneuploid cell populations (23%). In a separate cohort study of 322 patients with BE, DNA content tetraploidy (4N fractions > 6%) and aneuploidy were highly predictive of progression from BE to EA. The five year cumulative incidence of EA in patients who had normal flow cytometric results at the baseline endoscopy was only 5% compared to 62% for those with DNA content tetraploidy and 41% with aneuploidy.⁴² In patients without HGD, the five-year cumulative EA incidence was 28% in those with either DNA content tetraploidy or aneuploidy compared with 0% in those whose baseline flow cytometric results were normal.⁴² In a subsequent analysis of the same cohort, receiver operating characteristic (ROC) curves and two-sample log rank test statistic were used to determine the threshold of 6% for abnormal 4N fractions in BE.¹²¹ The distribution of aneuploid DNA contents was also reported to be bimodal with a near-diploid population less than or equal to 2.7N that appeared to have a lower rate of progression to EA.¹²¹ The five-year cumulative incidences of EA were 75% in patients with both aneuploidy greater than 2.7N and elevated 4N fractions compared to 5.2% in those with neither.¹²¹

Longitudinal studies with EA outcomes have also been reported for 17p LOH in BE. In 2001, a prospective cohort study of 269 patients with BE reported that those with 17p LOH had a 38% three-year cumulative incidence of EA compared to 3.3% among those with two 17p alleles.¹²² Patients with 17p LOH were also at increased risk of progression to DNA content tetraploid (RR=6.1) or aneuploid (RR=7.5) cell populations compared to those with two 17p alleles at the baseline endoscopy. This result was consistent with earlier studies in which 17p LOH was reported to develop in diploid cell populations before tetraploidy and aneuploidy.^{116, 123} In 2003, Dolan et al. reported a follow up study of 48 patients in endoscopic biopsy surveillance for BE.¹²⁴ Six of the 48 had 17p LOH detected in endoscopic biopsies; one progressed to EA and the other five to surrogate dysplasia endpoints (one HGD and four LGD). These studies suggest that measures of chromosome instability may be used for risk stratification in BE, but no single biomarker is likely to be adequate for clinical use. For example, a minority of cases of aneuploidy develop without 17p LOH, probably by an alternative pathway of chromosome instability.¹²²

In 2007, results of a 10-year prospective biomarker study in BE were reported using EA as the endpoint.¹¹⁷ In this study, 243 patients with BE were evaluated at a baseline endoscopy for the biomarkers and followed prospectively to determine which biomarkers were robust predictors of progression from BE to EA. The study was based on biomarkers that had been evaluated spatially in BE and included the chromosome instability biomarkers 9p LOH, 17p LOH and DNA content abnormalities (tetraploidy and aneuploidy) as well as the gene specific biomarkers TP53 mutation and CDKN2A mutation and methylation. At 10 years, all biomarkers except CDKN2A methylation and mutation contributed significantly to risk in univariate analysis. The chromosome instability panel of 9p LOH, 17p LOH and DNA content abnormalities (tetraploidy, aneuploidy) was the best predictor of EA among the biomarkers evaluated in the study (RR=38.7; 95% CI = 10.8-138.5; p<0.001) (Figure 2A). The five-year cumulative incidence of EA was 79.1% in patients with 9p LOH, 17p LOH and a DNA content abnormality at baseline. Patients with no biomarker abnormalities at baseline had a zero percent cumulative incidence of EA to nearly eight years.

Several recent studies have suggested that panels of SNPs or BACs can provide robust assessment of chromosomal instability using a single platform rather than the constellation of technologies used in the ten-year prospective study of LOH and flow cytometric DNA content abnormalities for clinical EA risk stratification in BE.^{33, 103} For example, flow cytometry is CLIA approved as a diagnostic test, but STR detection of LOH is not. In a recent advance, a SNP platform was designed for assessing LOH on 9p and 17p using a panel of SNPs that are closely linked to and have similar informativity to the STRs used in the 10 year prospective study.¹²⁵ In another recent study, 33K multisample SNP arrays were investigated as a common platform for assessing LOH and copy number using whole, unprocessed biopsies from BE.¹⁰³ The results indicated that the SNP array could detect LOH in whole biopsies and that genome-wide assessment of LOH or copy number provided robust discrimination between early and advanced BE/EA in the selected patient subsets with ROC areas under the curve of 0.91 for both LOH and copy number. A threshold of >1,000 SNPs accurately discriminated flow cytometric DNA content aneuploidy and detected five advanced cases that were classified as diploid by DNA content flow cytometry, which, at best, detects DNA content changes of 10% or more of the genome. Only a small number of chromosome abnormalities reached statistical significance in both early and late stages of progression. Small regions of copy loss or LOH were observed in fragile sites, many of which have been previously characterized in BE.^{90, 102, 126} There are several possible hypotheses for these early changes. (1) The early, high-frequency events might be necessary, but not sufficient for progression to EA. (2) The early changes might be selected as part of the early adaptation to chronic gastroesophageal reflux, unrelated to progression, but are carried into EA as hitchhikers (“passengers”) on later clonal expansions. (3) They might represent regions susceptible to chromosome damage, such as fragile sites, that undergo clonal expansion as hitchhikers on selected abnormalities such as an epigenetically modified progenitor population. Regardless, alterations that occur in these small regions both early and late in BE are far too common in early stages to be sufficient for development of EA because of the fact that rate of progression from BE to EA is low.^{17, 18}

A recent BAC array study reported that copy number provided a robust assessment of DNA content flow cytometric aneuploidy in 174 samples from a cohort of 98 patients with BE or EA.³³ In this study, the vast majority of flow cytometric DNA content diploid samples (141/155; 91%) had less than 180 BAC alterations, compared to 0/19 aneuploid samples. Using an empirical thresholding method, a threshold of 760 BACs with copy number alterations (CNAs) allowed identification of aneuploid samples with a sensitivity and specificity of 93% and 98%, respectively. Patients whose biopsies contained CNAs involving more than 70 Mbp had a significantly increased risk of progressing to DNA content abnormalities or EA during follow-up (HR=4.9, 95% CI 1.6-14.8, p=0.0047). These preliminary studies suggest that SNP platforms may provide a robust clinical assay for chromosome instability measures of LOH and copy number for risk stratification and early detection in BE.

In summary, a large body of research from many laboratories has provided evidence that chromosomal instability leading to copy gain, loss, LOH and DNA content abnormalities is associated with progression from BE to EA. A small fraction of EAs may manifest

microsatellite instability (MSI), but the numbers are sufficiently small that no single institution has been able to obtain enough cases to examine it robustly.^{127, 128}

Two recent studies have suggested that measures in peripheral blood cells associated with chromosome instability are associated with progression from BE to EA. Chao et al. measured mutagen sensitivity to bleomycin in peripheral blood lymphocytes in a cohort of 220 patients with BE who were followed for 1,230 person-years.¹²⁹ Bleomycin sensitive patients were found to be at increased risk of progression to aneuploidy (adjusted hazard ratio, 3.71; 95% CI 1.44-9.53) and nonsignificantly greater risk of cancer (adjusted hazard ratio, 1.63; 95% CI 0.71-3.75). Among patients with detectable 17p LOH including the TP53 locus, increasing bleomycin sensitivity was associated with increased risk of developing EA ($p(\text{trend}) < 0.001$) and aneuploidy ($p(\text{trend}) = 0.005$). This study supports the hypothesis that sensitivity to mutagens increases the risk of neoplastic progression in persons with Barrett's esophagus, particularly those with 17p LOH including the TP53 locus.

In a second study, telomere length was measured in baseline blood samples of a cohort of 300 patients with BE followed for a mean of 5.8 years.¹³⁰ Shorter telomeres were associated with increased EA risk (age-adjusted HR between top and bottom quartiles of telomere length, 3.45; 95% CI 1.35-8.78; $p = 0.009$). This association was still significant when adjusted for age, gender, nonsteroidal anti-inflammatory drug (NSAID) use, cigarette smoking, and waist-to-hip ratio (HR, 4.18; 95% CI 1.60-10.94; $p = 0.004$). The relationship between telomere length and EA risk was particularly strong among NSAID nonusers, ever smokers, and patients with low waist-to-hip ratio. These results demonstrate the ability of a test in peripheral blood, leukocyte telomere length, to predict the risk of future EA in persons with BE.

CHROMOSOME INSTABILITY AND USE OF ASPIRIN AND OTHER NSAIDS

A protective association of aspirin and other NSAIDs for risk of EA has been reported in case-control, cohort and meta-analyses.¹³¹⁻¹³⁴ In 2005, a prospective cohort study of 350 individuals with BE followed for 20,770 person-months reported that current users of aspirin and other NSAIDs at baseline and follow-up had a hazard ratio of 0.20 (95% CI 0.1-0.41) for progression to EA when compared to never users.¹³⁵ Current users also had reduced progression to DNA content aneuploidy (0.25; 95% CI 0.12-0.54) and tetraploidy (0.44; 95% CI 0.22-0.87) compared to never users. Use of aspirin and other NSAIDs was also assessed in the 10-year prospective biomarker study described above¹¹⁷ (Figure 2B). In that study, current use was associated with a marked risk reduction especially in patients with multiple chromosome instability abnormalities at baseline. In patients with zero, one, two or three chromosome instability biomarkers (9p LOH, 17p LOH, DNA content abnormality), there was a significant trend towards EA risk reduction in NSAID users compared to nonusers ($p=0.01$). The strongest protective association was observed in patients with multiple chromosome instability biomarkers with NSAID non-users having a 79% 10 year cumulative incidence of EA compared to 30% for NSAID users ($p<0.001$).¹¹⁷ Inhibition of COX-2 has also been reported to decrease the incidence of EA in an animal model of BE.¹³⁶

EPIGENETIC CHANGES IN BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA

Feinberg et al. have recently proposed a model of epigenetic progression in neoplasia.¹³⁷ In this model, progenitor cells develop epigenetic changes leading to an expanded and/or epigenetically altered progenitor cell pool that develops alterations in oncogenes, gatekeeper mutations or tumor suppressor genes to produce a benign neoplasm. Continued epigenetic and genetic plasticity then drive further neoplastic progression. There has been recent interest in epigenetic mechanisms, especially DNA methylation, in BE and EA, and the promoter regions of about four dozen genes have been evaluated using a candidate gene approach imported from other cancers.¹³⁸⁻¹⁵⁵

There has been some progress in investigating epigenetic abnormalities across spatial scales in BE and EA. Eads et al. reported the distribution of “fields” of methylation of *CDKN2A*, *APC* and *ESR1* in esophagectomy specimens of BE resected for EA.¹³⁹ *ESR1* methylation was found in inflammatory reflux esophagitis and all subsequent stages whereas *APC* and *CDKN2A* methylation were found in BE metaplasia, dysplasia and carcinoma. The authors concluded that when hypermethylation of *APC*, *CDKN2A*, and *ESR1* occurs, it is usually found in a large contiguous field, suggesting either a concerted methylation change associated with metaplasia or a clonal expansion of cells with abnormal hypermethylation. Wong et al. examined the spatial distribution of *CDKN2A* abnormalities, including methylation, mutation and 9p LOH, in endoscopic biopsies and concluded that both genetic and epigenetic changes were consistent with clonal expansions in the BE segment.¹¹⁵ To this author's knowledge, no other epigenetic abnormalities have been evaluated relative to genomic abnormalities in spatial scale experiments in BE or EA.

Longitudinal studies of epigenetic abnormalities have thus far met with limited success, in part perhaps because genome-wide scans have been unavailable, limiting discovery of novel methylation patterns and, in part, perhaps because the preliminary spatial scale experiments that might guide longitudinal studies have not been performed. Schulmann et al. examined methylation status of the promoters of 10 genes in 64 normal squamous esophagus, 93 BE and 77 EA in patients but the cohort from which these patients were selected was not well described.¹⁵⁶ Similar design limitations can be seen in a subsequent multicenter study of methylation and clinical findings as predictors of progression in BE.¹⁵⁷ In contrast to the advances in genomic-based biomarkers of progression, epigenetic studies have relied excessively on dysplasia classification, hardwiring the limitations of dysplasia classification into the epigenetic biomarkers.^{156, 157} The second study relied heavily on HGD as a surrogate endpoint HGD,¹⁵⁷ which does not accurately reflect health related endpoints of EA, EA mortality and all cause mortality.^{42, 43, 45-47} Use of surrogates such as HGD also increases the possibility of false-positive “progressors” due to diagnostic misclassification as a result of observer variation in diagnosis.^{39, 40, 48}

Until recently, technology has been limited to study DNA methylation on a genome-wide scale, and studies in BE and EA have generally been limited to promoter regions of small numbers of genes. However, several recent large studies have used unbiased scans of the genome to investigate DNA methylation in different tissue types and in some cancers.^{158,159}

The first comprehensive genome-wide set of tissue specific differentially methylated regions that may be involved in cellular identify and regulation of tissue specific genome function was reported.¹⁶⁰ This study investigated DNA methylation profiles of promoter regions, nonpromoter CpG islands, association with chromatin signatures, tissue specific differentially methylated regions (tDMRs), including promoter and gene-body tDMRs and their correlation with gene expression as well as Gene Ontology analysis of genes associated with promoter tDMRs. A recent study of the human colon cancer methylome using unbiased array-based relative methylation analysis reported that most methylation changes were not in promoters or CpG islands, but rather in sequences up to 2kb distant, termed “CpG island shores.”¹⁵⁸ There was significant overlap between locations of cancer methylation related changes and tissue specific methylation changes, consistent with the epigenetic progenitor model of cancer. The relative roles of DNA methylation in tissue differentiation and progression in BE are presently unknown because so few genes have been evaluated. Combining recent advances in genome-wide screens for epigenetic abnormalities, spatial scale experiments, and proper study design will likely lead to better understanding of the roles of methylation in tissue maintenance and neoplasia in BE and EA.^{158, 159}

OTHER ABNORMALITIES IN EARLY BE

BE has been reported to have abnormal proliferation compared to normal upper GI epithelia by a wide variety of techniques for nearly three decades. These measures include tritiated thymidine incorporation, flow cytometry, multiparameter flow cytometry, immunohistochemical staining with Ki67, PCNA, minichromosome maintenance proteins, and cyclins.^{77, 161-170} It has been hypothesized that assessment of cell cycle abnormalities may provide an integrated readout of loss of G1/S regulating tumor suppressor genes.¹⁷¹

Several longitudinal studies have been published using markers of proliferation. In 2000, Bani-Hani, et al. reported a nested case control study of cyclin D1 and TP53 immunohistochemistry of 12 patients with BE who progressed to EA from a cohort of 307 BE patients.¹⁷² They reported that patients whose biopsies were cyclin D1 positive were at significantly increased risk of progression to EA compared to those whose biopsies were negative (OR = 6.85; 95% CI = 1.57-29.91). Patients whose biopsies were TP53 immunopositive did not have a significantly increased risk of progression to EA (OR = 2.99; 95%CI = 0.57 – 15.76). In 2006, Murray et al. reported a larger nested case-control study of 29 patients who progressed to EA and six who progressed to the surrogate endpoint of HGD with up to five controls per case matched on age (within five years), gender and year of diagnosis. Control follow-up was at least as long as their respective case.¹⁷³ They assessed TP53, cyclin D1, COX-2, and β -catenin immunostaining and reported that TP53 positive staining was associated with an increased risk of progression (OR = 11.7; 95% CI= 1.93-71.4), but that the sensitivity was too low as a single biomarker to guide endoscopic surveillance. In this case-control study, there were no significant associations between cyclin D1, COX-2 and β -catenin immunostaining and progression.

Some studies have evaluated immunohistochemical measures of cell cycle or proliferative abnormalities using both cross-sectional and small case-control designs.^{168, 170} In one study, minichromosome maintenance proteins were evaluated in a cross-sectional study of

dysplasia grades and a small case-control study with nine EAs and 18 controls matched for age and BE segment length.¹⁶⁸ In the cross-sectional study, there was a statistically significant increased surface expression of Mcm2 with advancing grade of dysplasia with overlap between grades. In the case-control study, all patients progressed through the sequence of negative for dysplasia, LGD, HGD before developing EA. Mean Mcm2 surface expression was higher in cases than controls although the study design did not examine the full spectrum of risk because it excluded any controls that had multifocal LGD at any time in their surveillance program. Another report evaluated surface expression of cyclin A in both a cross-sectional study of dysplasia grades and a small case-control study with seven surrogate HGD endpoints and nine EA endpoints.¹⁷⁰ There was a statistically significant increase in cyclin A surface expression with advancing grades of dysplasia. The cases and controls were highly selected; all cases advanced through the negative, LGD, HGD sequence and nine developed EAs whereas controls had at least three surveillance endoscopies with no more than a single diagnosis of LGD. In this highly selected population, it was not possible to match for age or gender. The percentage of cyclin A positive surface cells was higher in the cases at all time points relative to the controls. However, the highly selected populations used in the study may make it difficult to extend the results to the general population of BE with a full spectrum of risk.

Recently a prospective cohort study (mean 6.3 years; 1,752 person years) of cell proliferation fractions and cell cycle intervals (G1, S, 4N) as well as the status of the G1/S regulatory tumor suppressor genes CDKN2A and TP53 was reported.¹⁷¹ Cell proliferative and cell cycle fractions were assessed in 853 diploid biopsies from 352 patients with BE. Higher total proliferative or G1 fractions were not associated with progression from BE to EA although increased S phase fractions were weakly associated with progression to EA ($p=0.03$) and 4N fractions were strongly associated with progression ($p<0.0001$), consistent with a previous report using DNA content flow cytometry.^{42, 121} CDKN2A and TP53 abnormalities were assessed in a subset of these biopsies in a cross-sectional study. Diploid S and 4N fractions were significantly higher in biopsies with TP53 mutation and LOH, and biallelic inactivation of TP53 was highly associated with elevated 4N fractions, which has been previously associated with progression to EA.¹²¹

CLONAL EVOLUTON AND NEOPLASTIC PROGRESSION IN BARRETT'S ESOPHAGUS

In 1976, Nowell articulated the theory of clonal evolution in neoplastic progression using a broad range of published data from the scientific community that included a variety of neoplasms ranging from CML to advanced, drug-resistant metastatic malignancies.¹⁷⁴ Nowell's model described generation of variants by genomic instability with natural selection operating on the variants to drive clonal expansions and extinctions. Nowell's insight on generation of variants, selection, and expansions of clones having selected mutations is in contrast to many of the genetic progression models of the 1990s, including some of those for BE, that were based on frequencies of abnormalities in different patients at different stages of progression.¹⁷⁵ However, studies across space and, to a lesser extent, time

scales have consistently highlighted the importance of clonal diversity and clonal expansions in BE (Figure 3).

Studies across space have included those in single cells, crypts, biopsies and representative sampling of the entire neoplasm. The earliest studies were of endoscopic and surgical biopsies obtained from defined regions of the Barrett's segment and evaluated by DNA content flow cytometry.¹¹⁸ Early "maps" of these results revealed striking heterogeneity of aneuploid cell populations in the BE mucosa surrounding early EAs in resection specimens, which was interpreted as genome-wide instability with generation of variants during neoplastic progression.¹¹⁸ Subsequent studies evaluated non-random LOH, including 5q, 9p, 13q, 17p, and 18q, DNA content abnormalities, CDKN2A mutation and methylation, and TP53 mutation in endoscopic biopsies and esophagectomy specimens.^{104, 105, 114-116, 176} These studies performed at the biopsy and BE segment spatial scales reported evidence of clonal heterogeneity and clonal expansions involving large regions of the esophageal mucosa with 9p LOH, 17p LOH and DNA content abnormalities occurring in the premalignant BE surrounding EAs.^{104, 105, 113, 115, 118, 123, 177}

A subsequent spatial study addressed the question of selected and neutral mutations in BE using clonal expansion as a measure of selection.¹⁰⁶ In this study of 211 persons with BE, CDKN2A mutation and methylation, 9p LOH, TP53 mutations and 17p LOH were all highly selected as evidenced by their association with clonal expansions. In contrast, LOH involving microsatellites on the long arms of chromosomes 9 and 17 and all microsatellite shifts behaved as neutral mutations that were not associated with clonal expansions. In some cases, nonselected neutral mutations were observed to undergo large clonal expansions, but these expansions could typically be explained by co-expansion of a known selective mutation. For example, 92% of microsatellite shifts that expanded could be explained as hitchhikers ("passengers") on selected CDKN2A abnormalities.

There has been debate in the literature concerning the relative importance of clonal expansions and genetic instability.¹⁷⁸⁻¹⁸³ This was addressed in a study that evaluated biopsies from 267 research participants with BE who had a mean 4.4 years of prospective follow-up, including 36 who progressed to EA.⁶ The sizes of clones with TP53 LOH (RR=1.27^x for an x cm clone; 95%CI = 1.07-1.60) or an abnormal DNA content (tetraploidy and aneuploidy) (RR=1.31^x for an x cm clone) increased the risk of progression from BE to EA. However, the sizes of CDKN2A clones were not significant risk factors for progression when TP53 LOH is accounted for in the model. These results suggest that the combination of clonal expansion and genetic instability interact to promote progression from BE to EA and likely integrates assessment of increased population size with mutation rate.

Much research has focused on tumor suppressor genes, oncogenes and the genes associated with hallmarks of cancer,¹⁸⁴ but fewer studies have addressed evolutionary dynamics in populations of cells during neoplastic evolution as illustrated in Nowell's model.^{174, 185} For example, it is unknown whether a clonal expansion that homogenizes a cell population as is seen in some CDKN2A expansions in BE¹¹⁵ or accumulation of clonal diversity as suggested with emergence of multiple aneuploid populations in BE¹¹⁸ is more predictive of progression to cancer. This was evaluated in a cohort study of 268 patients with BE

followed prospectively with 37 EA outcomes.¹¹⁹ Increased clonal genetic diversity as assessed by a number of measures, including number of clones, Shannon Index and mean pairwise genetic divergence were all associated with increased risk of progression from BE to EA even when controlling for TP53 LOH and DNA content tetraploidy and aneuploidy.¹¹⁹ The association of clonal diversity with progression to EA provided an evolutionary mechanism for clonal progression but did not address mechanisms for generating cellular heterogeneity. FISH probes to chromosome 17 centromere and TP53 locus on 17p were used to assess cellular diversity in BE with and without TP53 LOH. Diversity, as assessed by Shannon Index, was higher in biopsies with TP53 LOH than in those without, indicating that TP53 LOH is also associated with increased cellular diversity.

An interesting paper recently investigated diversity at the crypt level in BE.¹¹² The investigators dissected individual crypts from BE segments and evaluated them for LOH involving APC (5q), p16 (9p) and TP53 (17p) using microsatellite polymorphisms as well as p16 and TP53 point mutations. The samples included six endoscopic biopsies from five patients and four esophagectomy blocks each from two patients and two blocks from a third patient. They reported that dissection across esophagectomy specimens revealed marked heterogeneity among crypts. In addition, in one patient they reported a noncoding p16 mutation that was present in both a squamous esophageal duct and metaplastic BE, suggesting that the duct might be the origin of BE in this case. The careful attention to spatial scale in this manuscript advances our understanding of levels of heterogeneity in BE.

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“This paper concerns a condition whose existence is denied by some, misunderstood by others, and ignored by the majority of surgeons. It has been called a variety of names which have confused the story because they have suggested incorrect etiological explanations...” Norman Barrett, 1957

“Cancer is a disease of the genome.” Ruth Sager, 1985

Acquired genetic lability permits stepwise selection of variant sublines and underlies tumor progression.” Peter Nowell, 1976

Figure 1. Endoscopic biopsies stained with hematoxylin and eosin at 200 magnification
A) stratified squamous epithelium in normal esophagus and B) intestinalized specialized metaplasia in Barrett's esophagus. (Digital images courtesy of Robert D. Odze, MD, Brigham and Women's Hospital, Boston Massachusetts.)

Figure 2A. Cumulative EA incidence with combinations of chromosomal biomarker abnormalities (17p LOH, DNA content abnormality, 9p LOH)

Cancer incidence rates are shown for all participants with no selected abnormalities (17p LOH, DNA content abnormalities, or 9p LOH) at baseline (red), any one abnormality (green), any combination of two abnormalities (blue), or all three abnormalities (black).¹¹⁷

Figure 2B. Modulation of EA risk by NSAIDs in participants with different baseline abnormalities

Patients are classified according the number of chromosomal biomarker abnormalities, 17p LOH, 9p LOH or any DNA content abnormality (aneuploidy and/or tetraploidy) at baseline. The top two curves represent > 1 baseline abnormality and the lower two curves represent ≤ 1 abnormality. Shown are Kaplan Meier curves of cancer incidence rates in patients who are NSAID non-users (former or never users) (red) or NSAID users (black).¹¹⁷

Figure 3. Clonal evolution in Barrett's esophagus

The X axis represents time, and the Y axis represents the extent of the Barrett's segment length. BE arises in a subset of patients in response to the harsh environment of gastroesophageal reflux. Loss of one or both alleles of CDKN2A provides a selective advantage leading to clonal expansion. Neutral mutations also occur during clonal evolution. A neutral mutation arising in a clone with a selected mutation such as those affecting CDKN2A can expand as a hitchhiker on the selected mutation. Otherwise, neutral mutations expand or contract through a random process of genetic drift. TP53 mutations and LOH are selected as later events in neoplastic evolution, almost exclusively in the genetic background of CDKN2A variants. TP53 variants have pleiotropic effects including loss of cell cycle checkpoint control, evasion of apoptosis, and genomic instability that increase genetic diversity within the neoplasm, and they are permissive for subsequent evolution of tetraploid and aneuploid populations. The sizes of genetically unstable clones with TP53 abnormalities and aneuploidy are predictive of future progression to esophageal adenocarcinoma. A panel of chromosomal instability biomarkers (9p, 17p LOH, tetraploidy, aneuploidy) provides independent cancer risk prediction in BE, but mutations in CDKN2A and TP53 and methylation of the CDKN2A promoter do not. A) Illustration of a Barrett's segment with CDKN2A abnormalities with neutral and hitchhiker mutations. B) Illustration of a Barrett's segment with expansion of a CDKN2A clone, followed by evolution of clones with TP53 mutation and 17pLOH that is permissive for evolution of aneuploidy and progression to cancer.