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The Molecular Evolution of Metaplasia to Carcinoma in the Esophagus

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Abstract

Esophageal adenocarcinoma (EAC) develops from Barrett's esophagus (BE), a condition where the normal squamous epithelia is replaced by specialized intestinal metaplasia in response to chronic gastro-esophageal acid reflux. In a minority of individuals, BE can progress to low- and high-grade dysplasia (LGD and HGD) and eventually to intramucosal and then invasive carcinoma. BE provides researchers with a unique model to characterize the process by which a carcinoma arises from its precursor lesion. Molecular studies of BE have demonstrated that it is not simply a metaplastic tissue, but rather it harbors frequent alterations that are also present in dysplastic BE and in EAC. Both BE and EAC are characterized by loss of heterozygosity (LOH), aneuploidy, specific genetic mutations, and clonal diversity. Epigenetic abnormalities, primary alterations in DNA methylation, are also frequently seen in BE and EAC. Candidate gene and array-based approaches have demonstrated that numerous tumor-suppressor genes exhibit aberrant promoter methylation, and some of these altered genes are associated with the neoplastic progression of BE. It has also been shown that the BE and EAC epigenomes are characterized by hypomethylation of intragenic and non-coding regions. Recent studies have also provided new insight into the evolutionary forces underlying the molecular alterations seen in BE and EAC and into the molecular pathogenesis of EAC.

Keywords

Barrett's esophagus; esophageal adenocarcinoma; cancer genomics; LOH; aneuploidy; genomic instability; DNA methylation

INTRODUCTION

EAC develops from Barrett's esophagus (BE), an intestinal metaplasia of the lower esophagus, which can progress through low- and high-grade dysplasia (LGD and HGD) to intramucosal carcinoma and invasive carcinoma, presumably in response to chronic gastroesophageal reflux^{1–3}. Several concurrent histologic and molecular changes have been

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described for BE and EAC⁴⁻⁷. The molecular changes observed include structural genomic alterations (amplifications and deletions, translocations), DNA sequence alterations (e.g. missense mutations), and epigenetic modifications, primarily in the form of DNA hypermethylation and hypomethylation of CpG dinucleotides, and are widely believed to be a principal mechanism that drives the initiation and progression of BE and EAC. Since the initial discovery of genetic alterations in BE and EAC, our understanding of the molecular evolution of EAC has progressed from one that only accounted for sequence alterations in the pathogenesis and was uniform between all cases of BE and EAC to one that recognizes the role of epigenetic alterations and the tumor microenvironment as well as inter- and intra-lesional heterogeneity. This evolving understanding of the molecular pathogenesis has been informed not only by the direct study of BE and EAC using state of the art genome-wide analysis methods but also by the study of other inflammation-associated cancers (e.g. H pylori induced stomach cancer, colitis associated cancer). Inflammation appears to create a permissive state for cancer formation by promoting mutations and creating a microenvironment that cooperates with certain DNA alterations to drive tumor progression⁸. Other host related factors that increase the risk of EAC (e.g. obesity, diabetes mellitus, etc.) also may affect the molecular evolution of BE and EAC by providing factors that promote the clonal selection of tumor stem-like cells that have acquired favorable DNA alterations, such as *TP53* mutations, which are highly correlated with progressed BE and EAC.

An important point related to this discussion of host factors, tumor stem cells, and clonal evolution in the BE→EAC progression sequence is that the cell of origin of BE and EAC is still unknown, but likely is either gastric cardia, squamous esophageal epithelium or cells from the esophageal appendages. The significance of the identification of the cell of origin lies in the insights this will bring related to the biological impact of gene mutations on the transformation process, which are predicated by the underlying ground state of the cell. Another consideration that is important to recognize is that as with all cancers studied to date using modern methods, there is substantial intra-lesional and inter-lesional heterogeneity in genetic and epigenetic alterations. When intra-lesional clonal heterogeneity arises in the BE→EAC sequence and the role it plays in transformation is under active investigation. Some studies have shown BE progression is associated with increasing clonal diversity and that there is the potential for certain clones to become dominant over time, i.e. a 'clonal sweep'^{7, 9, 10}.

Thus, these host factors in combination with the serial, but not necessarily linear, accumulation of genetic and epigenetic alterations are currently believed to underlie the molecular evolution of EAC and will be discussed further in the remaining sections.

Models of evolution of alterations in BE and EAC and relationship to molecular alterations

The development of a malignant clone appears to involve a process that follows the principles of Darwinian evolution¹¹⁻¹³. In this process, a single tumor-initiating clone, instead of the whole organism, is what is being subjected to evolutionary forces. The environment that applies the selection pressure is the tissue microenvironment surrounding the single clone. Somatically acquired variation, instead of inherited variation fuels this evolutionary process and our knowledge of this process has garnered through studying the

final product of this process: the cancer epi/genome. The discovery of gene mutations in the DNA of cancer cells opened up the field of cancer molecular genetics and led to a prevailing model of tumorigenesis, which entails the gradual accumulation of gene mutations and epigenetic alterations that drive processes important for tumor initiation and progression. These DNA alterations perturb signaling pathways and generate mutant proteins that induce oncogenic behavior in the evolving cancer cell. Through a process of Darwinian evolution and clonal selection, those cells that obtain the greatest growth advantage become the prominent cells in the tumor. Thus, the serial accumulation of genetic and epigenetic alterations has been believed to mediate the histologic steps of cancers, including EAC.

The classic model of the molecular evolution of EAC described above has been under careful review in the last 10 years because of the availability of genome wide, highly detailed DNA sequence data and DNA methylation data. This has revealed substantial intra-tumor and inter-tumor heterogeneity as well as the presence of gene mutations and epigenetic alterations in pre-cancerous lesions. Emerging data is demonstrating that there are multiple different modes of molecular evolution in the BE→EAC sequence. It is unclear at this time which model is the most prevalent. We will discuss these models, which include punctuated equilibrium, the Big Bang model, classic cellular Darwinian evolution (aka gradualism), and catastrophic genetic events, such as chromothripsis, in more detail below.

Gradualism

According to this model, cancers in the esophagus are believed to arise via the gradual stepwise accumulation of mutations¹⁴. The progression from normal tissue to specialized intestinal metaplasia, followed by low-grade and high-grade dysplasia, finally locally invasive cancer, and then metastatic cancer is thought to be driven by the accumulation of mutations that perturb specific genetic pathways at each step in the tumorigenic process. At each step, a new mutation or epigenetic alteration is thought to generate a sub-clone with the most advantageous mutation profile and a strong selective advantage, outcompeting less fit clones in a Darwinian fashion and prompting progression to the next stage of the disease process. Under this model, all BE lesions are believed to have the potential to become EAC and the risk of EAC is increased by the accumulation of cooperating mutant and epigenetically altered genes, which occurs in a stochastic fashion.

Big Bang Tumor Evolution Model

Other models of cancer evolution have been proposed since the initial concept of gradual Darwinian evolution in cancers. One alternative model of cancer evolution is the “Big Bang” theory of tumorigenesis¹⁵. In this model, the majority of mutations seen in EAC’s arise during the first few neoplastic cell divisions that establish BE. This high rate of mutations early in the tumorigenesis process generates many passenger mutations and copy number alterations. This initial spurt of mutations and resulting increased evolutionary tempo may be an intrinsic aspect of neoplastic transformation or may be a consequence of a critical genetic or epigenetic event that leads to genomic instability and increased rates of DNA alterations (e.g. mutations, chromosomal rearrangements, etc.)^{16,17,18}. Once all the necessary driver mutations are acquired, cancers grow from a single expansion of a diverse population of tumor cells, characterized by neutral evolution instead of Darwinian survival^{19–22}. This

model accounts for the presence of intratumoral heterogeneity in EACs that is not well explained by the gradualism model. In the Big Bang growth model, intratumoral heterogeneity arises as a function of time, becoming detectable upon analysis when the population has expanded to a sufficient size, and not as a function of increased fitness. Therefore, the most recently acquired mutations will be at the lowest frequencies in the population, often undetectable by bulk genomic methodologies.

Importantly because of the clinical implications, the Big Bang growth model can account for variation in tumor growth and progression fates between different cases of BE. Sets of mutations that are acquired early at the Big Bang event can contribute to the fate of BE, allowing for an accelerated growth rate if the right combination of alterations are present. This concept of some tumors being “born to be bad” attempts to explain those interval cancers and cases of BE that progress faster than the predicted time of 10–20 years²³ While the Big Bang growth model seeks to better explain some of the variation across tumor growth and progression, it is only one evolutionary model and does not appear to apply to all EACs or cases of BE.

Punctuated Equilibrium

In addition to the Big Bang model, there is evidence from exome sequencing studies of cancer genomes to support punctuated equilibrium in cancer evolution^{24, 25, 26, 27} Punctuated equilibrium involves long periods of stasis, punctuated by rapid periods of transformation and molecular changes, which appears to occur in some cases of BE and EAC²⁸. The core processes involved in punctuated equilibrium are: 1) stasis, which is characterized by the neutral accrual of passenger mutations and no phenotypic changes; 2) gradualism, which is a stepwise growth model resulting from the serial accumulation of molecular alterations and natural selection resulting in BE→EAC progression; and 3) punctuation events defined by periods of genomic instability with many resulting molecular changes concurrently and a dramatic phenotypic change. Punctuated equilibrium or models that include elements of punctuated equilibrium can account for the variability across BE progression rates that occur among patients as well as the finding of intratumoral heterogeneity within BE and EACs.

A criticism of the gradualism classical stepwise acquisition model is the finding of intratumoral heterogeneity within both BE and EAC^{29, 30}. In the punctuated equilibrium model, multiple clones can co-exist due to the variation of when natural selection is active and when the majority of genetic and epigenetic alterations occur. Neither punctuation events nor stasis require natural selection to be in place to generate detectable mutations and can account for the expansion of neutral passenger mutations. An important aspect of punctuated equilibrium and stasis is that they can account for tumor progression without necessitating that a clonal sweep of the most malignant clone has occurred. Based on the Big Bang and punctuated equilibrium models, BE and EACs of any size can have different amounts of intratumoral heterogeneity. Overall, the cancer punctuated equilibrium model does appear to be the best fit with the anatomic and molecular observations during the BE→EAC progression sequence. However, the precise timing of the three mechanisms during the evolution of a malignant cancer as well as the dynamics between multiple clones

remains unknown and it is likely that all three models are true and apply to different subsets of BE and EACs.

Chromothripsis

Finally, we will note that some EACs display evidence of chromosomal catastrophes that result in the sudden accumulation of chromosomal breaks, gains, losses, and rearrangements. This process is termed chromothripsis, which refers to a massive “chromosome shattering” event and that in a fashion similar to punctuated equilibrium can lead to rapid progression of BE to EAC. This appears to occur in up to 30% of BE and EAC cases^{31–33}

The Molecular Process of Metaplasia-Dysplasia-Adenocarcinoma (MDA) sequence in the esophagus

Overview of the MDA sequence in the esophagus—The stepwise development of esophageal adenocarcinoma, termed the Metaplasia-Dysplasia-Adenocarcinoma (MDA) sequence, suggests a sequential progression from normal esophageal lining, to specialized intestinal metaplasia, dysplasia and ultimately adenocarcinoma in the esophagus³⁴. Although this model was proposed without much knowledge about the molecular factors associated with this process, this paradigm of clonal evolution of the EAC from the Barrett’s metaplasia is supported by recent studies that have uncovered the complexity of the BE epi/genome, and revealed common fundamental molecular alterations along multiple biological pathways from BE to EAC. These molecular changes contribute to the selection of a specialized Barrett’s metaplastic lineage, which tolerates chronic exposure to bile acid and refluxate. Subsequent epi/genomic alterations lead to oncogenic behaviors, such as proliferation advantage and inhibition of apoptosis in the metaplastic clones, which enables clonal expansion and development of malignancy as described earlier.

Genomic alterations during the Barretts Esophagus->Dysplasia->Adenocarcinoma sequence

Overview—The progression of BE to EAC has provided a unique system to characterize the process by which a carcinoma emerges from its precursor state. Genomic studies of BE have revealed that it is not simply a metaplastic tissue but that many BE lesions also harbor somatic genetic alterations and nearly all BE lesions have substantial epigenetic alterations when compared to the normal squamous esophagus or cardia^{29, 35–37}. The analysis of the molecular events involved in the process of BE progression has been greatly enhanced by dramatic improvements in genomic technologies, including tools to examine somatic nucleotide variants (SNVs; aka gene mutations), larger structural alterations, and epigenetic alterations (DNA methylation, microRNA expression) in cancer and pre-cancer genomes.

Genomic alterations in BE and EAC and potential role in the BE->Adenocarcinoma sequence

Early studies that used PCR-based targeted sequencing identified *TP53* mutations in exon 5–8 present in EAC and BE adjacent to EAC compared to corresponding normal squamous tissue from the resection margin³⁸. Further, specimens of Barrett’s epithelium from separate sites had identical *p53* mutations, suggesting a clonal origin. Frequent loss of heterozygosity

(LOH) at 17p, 5q, 9p, and 13q was found in established BE and EAC^{1, 2, 39}. 17p and 9p harbor the tumor suppressors *TP53* and *CDKN2A*, respectively, and studies have revealed frequent LOH through mutation (*TP53* and *CDKN2A*) or promoter methylation (*CDKN2A*). The 9p LOH event appears to occur prior to the onset of 17p LOH and is widely distributed throughout the BE lesion⁴⁰. 17p LOH is associated with genomic doubling to a 4N state, consistent with the impact of p53 loss upon genomic instability. These findings have led to a model where *CDKN2A* loss is thought to be an initiating event in BE progression, while *TP53* alterations are later events, associated with neoplastic progression and aneuploidy. These findings also provided evidence that, like colorectal cancer, the BE→EAC sequence involved the serial and gradual accumulation of genetic alterations that drive the initiation and progression of BE to cancer.

The landscapes and spectrum of genomic alterations in BE and EAC have been described in more recent studies using the next generation sequencing^{29, 33, 35, 41, 42}. Studies using whole genome sequencing and exome sequencing, which have thoroughly characterized the mutations in EAC and BE, have supported the accepted paradigm that EAC emerges from BE by showing that many coding mutations in EACs are already present in BE^{29, 35, 43, 44}. The most comprehensive large-scale sequencing study in BE samples to date analyzed 26 genes (selected because they are commonly mutated in EAC) in a collection of non-dysplastic BE (non-progressors), BE with HGD, and EAC samples³⁵. Remarkably, with the exception of *TP53* and *SMAD4*, there were no differences in the frequencies of specific gene mutations between BE and EAC, even for bona fide tumor suppressors such as *CDKN2A* and *ARID1A*. For *TP53*, only 2.5% of non-dysplastic BE contained a mutation but 70% of cases of HGD and EAC were *TP53* mutant. *SMAD4* mutations were only found in EAC (13%). It is notable that even non-dysplastic BE lesions from patients who have not progressed to EAC contained tumor suppressor inactivation raising new questions about how mutations and non-mutation cancer related phenomenon contribute to cancer formation.

Further insight into the role of genomic alterations was provided by Stachler and colleagues who observed that genome doubling appears to precede progression of EAC to BE in the majority of cases (62.5%) and that tumors with genomic doubling have more frequent oncogene amplification events and less frequent tumor suppressor gene inactivation compared to those without²⁹. These findings are consistent with other studies, which suggested that the BE-EAC progression sequence is not one characterized by the gradual, linear serial accumulation of mutations in oncogenes and tumor suppressor genes, but rather a non-linear process that leads to BE transformation after the sudden occurrence of genomic doubling and genomic alterations, possibly secondary to *TP53* mutations^{29, 32, 45}.

With regards to the study of genetic alterations in established EAC, substantial advances have occurred in the last five years secondary to next generation sequencing technology. EACs have relatively high somatic mutation rates compared to most other epithelial cancers^{32, 35, 41, 43}. Furthermore, Dulak et al performed whole exome sequencing on 149 EACs, along with whole genome sequencing of 15 of these EACs (Figure 1)⁴¹. Somatic mutations in 26 genes were identified to be implicated in EAC, affecting the Wnt/b-catenin, RTK-RAS-PI3K, TGF- β /SMAD4, chromatin-remodeling enzyme, G1-S cell cycle control and p53 pathways. Not all mutations are equally frequent. The top mutated gene in EAC is

TP53 (found in 72% of cases), followed by CDKN2A in 12% of EAC cases. Novel recurrent mutations, including those involving *TLR4* and *ELMO1* were also noted, but their pathologic significance remains to be defined. Dulak and colleagues were also able to evaluate mutation patterns, and found a predilection for A to C transversions at AA dinucleotides. The etiology of these mutations is unknown, but it has been hypothesized to be linked to bile acid exposure and the induction of oxidative DNA damage. This novel mutation signature was also observed in whole genome sequencing of esophageal cancers by other groups^{32, 35}. These studies have revealed the genetic alterations that are commonly present in EACs and BE and have now presented the challenge of determining which alterations are potential driver events during the BE→EAC sequence. These results have also revealed the inter-tumor heterogeneity in EAC. Thus the same histopathological diagnosis of these EAC cases appears to be a poor reflection of their molecular diversity.

Epigenomic alterations during the metaplasia to EAC tumor evolution

Overview—Epigenetics broadly refers to heritable and stable alterations in gene expression that are not mediated by changes in the DNA sequence. Since the discovery of DNA hypomethylation in colorectal cancer in 1982, epigenetic research has revealed an epigenetic landscape consisting of a complex array of epigenetic regulatory mechanisms that control gene expression in both cancer^{46, 47} and normal tissue⁴⁸. The epigenetic landscape affects gene expression through the chromatin condensation state, which regulate the availability of DNA to transcription factors, etc, as well as through chemical modifications of base pairs⁴⁸. The epigenetic mechanisms and marks commonly altered in cancer and currently believed to play a role in cancer include: 1) DNA methylation of cytosine bases in CG-rich sequences, called CpG Islands; 2) post-translational modifications of histones, proteins that form the nucleosomes, which regulate packaging of DNA in chromatin; 3) microRNAs and noncoding RNAs; and 4) nucleosome positioning⁴⁸. Aberrant DNA methylation is the best studied epigenetic alteration in cancer and will be the focus of the following discussion. A number of excellent publications focusing on other classes of epigenetic alterations, such as histone modifications, are available and the interested reader is directed to those reviews^{49–52}.

Aberrant DNA methylation in BE and EAC—Global alterations in DNA methylation are present in both BE and EAC. Numerous studies have assessed BE and EAC using loci/gene specific assays, methylation arrays or next generation bisulfite sequencing. Aberrant DNA methylation of promoter CpG islands, which can induce transcriptional repression of tumor suppressor genes, has been found frequently throughout the BE→dysplastic BE→EAC progression sequence. The majority of methylated genes/loci appear to arise during the formation of BE^{37, 53, 54}. One of the first tumor suppressor genes shown to be aberrantly methylated in 3–77% of BE cases is *CDKN2A* (*p16INK4a*), which normally blocks phosphorylation of the Rb protein and inhibits cell cycle progression^{55–58}. Eads et al evaluated methylation patterns of *APC*, *ESR1*, and *CDH1* in six esophagectomy specimens, which contained both BE and EAC. They analyzed 107 distinct regions of each resected specimen in order to create spatial methylation maps. They found a high incidence of methylation of *ESR1*, *APC* and *CDKN2A* in BE, BE with dysplasia, and EAC in a pattern suggesting simultaneous methylation in large contiguous fields, or clonal expansion of cells

that acquired methylation⁵⁸. Similar patterns consistent with clonal expansion in BE have been reported in studies that focused on LOH or mutations of *APC*, *TP53*, and *CDKN2A*^{3, 55, 59}. Since the discovery of aberrant DNA methylation in single or small numbers of genes in BE and EAC over 20 years ago, it has been shown that thousands of loci and genes are methylated in BE and EAC when compared to normal esophagus^{37, 53, 54}. Using HM450 methylation arrays, which can assess approximately 480,000 CpGs, Krause et al discovered methylation of >9000 CpGs in BE and EAC⁵⁴. Some of the genes are so commonly methylated that they have been shown to sensitive biomarkers for BE (e.g. *VIM*, *B3GAT2*, *ZNF793*, *TFIO2*, *TWIST1*, etc), while others are less frequently methylated (<5% of BE cases) demonstrating intersample heterogeneity^{60–62}. Only a subset of the aberrantly methylated genes show loss of expression in BE and/or EAC and the biological consequences of the DNA methylation is still being determined for the vast majority of genes^{42, 54, 63}. As with gene mutations, it is widely believed that most epigenetic alterations in neoplastic and malignant lesions are passenger events and that only a subset are drivers of the initiation and transformation process. In BE and EAC, most driver epigenetic alterations remain to be defined, with a few notable exceptions like *CDKN2A* and *PKP1*^{64, 65}.

Mechanisms causing BE and EAC related aberrant methylation—Despite the near universal observation of altered DNA methylation in BE and EAC, the mechanisms driving aberrant DNA methylation in the esophagus, as in most other pre-neoplastic and neoplastic tissues, remain elusive. A number of candidate mechanisms have some suggested from published studies and include age-related DNA hyper and hypo-methylation, mutations or changes in expression of genes regulating DNA methylation or chromatin states, and environmental factors^{66–69}. None of these candidate mechanisms has been clearly shown to drive methylation alterations in BE and EAC. One particularly interesting candidate mechanism that has received increased attention recently is age related DNA methylation, especially given the age related risk associated with BE and EAC. Interestingly, the methylation patterns at specific loci have been found to gradually increase or decrease in aging cells and tissues due to a process termed epigenetic drift, in which the epigenetic states are not maintained during DNA replication^{66, 70}. Assuming that epigenetic drift is a stochastic process, which couples to cellular turnover and tissue aging, it can be used as ‘biological clock’, a marker of the biological age of tissue. Such epigenetic ‘biological clock’ CpGs has been identified through a genome-wide assessment of DNA methylation in various types of tissues from individuals within diverse age groups^{71–73}. Using DNA methylation-based markers that quantitatively measure biological age in tissues, these studies collectively suggest biologically older tissues may be at higher risk of becoming cancer, presumably secondary to the age-related increased load of aberrantly methylated genes⁶⁶. Curtius et al., identified a set of 67 CpG dinucleotides that show significant age-related drift in BE tissue relative to normal squamous tissue and used them as BE-specific molecular clock to infer the time of BE onset⁷⁴. A similar pattern of methylomic drift was found in EAC and correlated with transcriptional repression of genes implicated in EAC biology, suggesting a functional role of extensive epigenetic drift during BE to EAC progression⁷⁵. Further studies of epigenetic drift and other candidate mechanisms of aberrant DNA methylation are still needed at this time.

Molecular subtypes of BE and EAC identified with distinct methylation

patterns—The molecular heterogeneity of cancer, including BE and EAC, is widely accepted now. This has led to efforts to identify distinct molecular subtypes of BE and EAC. Kaz et al were the first investigators to observe distinct methylation subtypes of BE and EAC using GoldenGate methylation arrays, which survey approximately 1500 cancer related CpGs. They found in a small set of BE and EAC cases (BE N=28; EAC N=29) low and high methylation epigenotypes ³⁶. More recently, an integrated genomic and epigenomic analysis of esophageal cancer from the Cancer Genome Atlas (TCGA) consortium found that EACs have a pattern of global DNA methylation alterations that resembles that observed in a subset of hypermethylated gastric cancers ⁴². However, whether the DNA methylation features found by the TCGA exist in BE is not clear yet. Krause et al found methylation profiles from HM450 methylation arrays revealed two EAC subtypes defined by the presence or absence of widespread CpG island hypermethylation overlapping H3K27me3 marks and binding sites of the Polycomb proteins. The subtypes were validated by an analysis of an independent set of 89 esophageal cancer samples. The most hypermethylated tumors showed worse patient survival ⁵⁴. Further studies are needed to determine if the methylation subtypes of EAC have unique sensitivities to cancer therapy or unique biological activities.

Alterations in MicroRNA Expression in Barrett's Esophagus and Esophageal

Adenocarcinoma—MicroRNAs are small noncoding RNA molecules which can interact with other RNA molecules, resulting in post-transcriptional regulation of gene expression and gene silencing ⁷⁶. Although most of the data regarding the role of miRNAs in esophageal cancer pertains to squamous cell carcinoma, there is evidence that *miR-21* and *miR-375* play a role in BE and EAC. Several studies have demonstrated that *miR-21* is upregulated in BE and EAC compared to the normal esophagus. Feber et al showed that miRNA expression profiles distinguished normal esophagus from EAC, and that *miR-21* expression was 3–5 fold increased in EAC compared to normal epithelia ⁷⁷. Other investigators using microRNA microarrays found 34 differentially expressed miRNAs between normal squamous epithelium and BE/EACs, although the miRNA profile did not reliably distinguish BE from EAC ⁷⁸. In a validation cohort, the five microRNAs chosen for validation with qRT-PCR, including *miR-21*, were successfully able to discriminate normal esophagus from BE/EAC.

There is also evidence that differential expression of miRNAs is associated with the progression of BE to EAC. Revilla-Nuin et al found 23 miRNAs involved in BE progression using miRNA sequencing analysis, finding four miRNAs (*miR-192*, *miR-194*, *miR-196a*, and *miR-196b*) had higher expression in BE patients who progressed to cancer compared to those who did not progress ⁷⁹. Functional studies in cell line systems have also found other candidate driver genes such as the nc-RNA, *AFAP1-AS1*, which is highly hypomethylated and overexpressed in BE and EAC tissues and cell lines⁸⁰. When *AFAP1* was silenced using siRNA technologies, esophageal cells exhibited increased apoptosis and reduced proliferation and colon-forming abilities, suggesting a cancer-promoting role for this noncoding RNA in BE and EAC.

In order to understand the implications and significance of genetic and epigenetic alterations in the BE→EAC sequence, it is important to recognize that the vast majority of patients with BE do not progress to cancer, making the contribution of specific genomic alterations to the process of carcinogenesis unclear without follow-up information regarding the fate of the patients with BE. There are only a few prospective studies of BE patients who did or did not progress to cancer. One of these is the Seattle Barretts Esophagus Study, which has been used to show progressors have chromosomal instability, genome doubling, and an increase in genetic diversity in BE samples taken within 48 months of EAC diagnosis compared to BE samples from non-progressors, which had relatively stable genomes with few copy number changes⁸¹. These results are consistent with the concept that aneuploidy associates with neoplastic progression, perhaps by accelerating the acquisition of transforming genetic and epigenetic alterations, and further showed that the aneuploidy was acquired just prior to the diagnosis of cancer.

Clinical implications

Use of molecular markers as biomarkers for diagnosis or surveillance—

Hundreds of potential diagnostic and prognostic biomarkers have been reported in the literature, but very few have been shown to be reproducible and robust⁸². There is one molecular marker that has sufficient validation data to support its consideration for clinical use—p53 immunohistochemistry. Nuclear expression of p53 is a surrogate for *TP53* mutations that stabilize the p53 protein and complete loss p53 can also indicate a *TP53* mutation that leads to failed translation of the protein. However, this assay is not 100% sensitive for all inactivating genetic alterations⁸³. Studies of nuclear p53 expression by IHC conducted in large observational cohorts of patients with BE have shown that this assay can improve inter-observer variability in diagnosing dysplasia and can predict progression risk with an OR of 3–8^{84,91}. However, concerns about the reproducibility of this assay remain as the p53 positivity rate in Barrett's esophagus dysplasia is variably reported in the literature, ranging from 50% to 89%^{87, 88}. This appears to be because p53 immunostaining protocols are not standardized nor is the interpretation of the results leading to inter-observer variability and suboptimal reproducibility for a clinical assay. Some authorities in this field have proposed that the addition of p53 immunostaining to the histopathological assessment may improve the diagnostic reproducibility of a diagnosis of dysplasia in BE and should be considered as an adjunct to routine clinical diagnosis. This has led to the British Society of Gastroenterology to propose its use in this setting (Grade C recommendation in the most recent BSG Guidelines)⁹².

Use of genomic instability as a biomarker for risk prediction of progression to

EAC.—Maley, Reid and colleagues have conducted numerous studies describing the relationship between clonal diversity and clonal expansions and the risk of BE progression^{7, 12, 93–97}. One prospective study of 268 BE patients evaluated whether clonal expansions during the progression of BE lead to homogenous cell populations or result in clonal diversity⁷. 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 diversity⁸¹. Individuals that progressed to EAC, meanwhile, 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 also assessment of multiple foci of BE from individual patients.

Conclusions

Barrett's esophagus is a metaplastic tissue that develops in response to chemical injury and is a major risk factor for EAC. The fact that many individuals with BE undergo periodic endoscopy with tissue biopsy means that a valuable source of material to study the molecular changes associated with BE, BE with dysplasia, and EAC is readily available. The molecular changes that have been identified to date include structural genomic alterations, DNA sequence alterations, and epigenetic modifications.

Genomic studies of BE have revealed that it is not simply a metaplastic tissue, but characterized by frequent somatic alterations, including mutations in *TP53* and other genes. BE is also characterized by aneuploidy and activation of oncogenes, both of which appear to be important precursors for progression to cancer. Newer sequencing technologies that have now been used to characterize EAC have demonstrated relatively high somatic mutation rates compared to most other epithelial cancers.

Epigenetic alterations are also frequently found in BE and EAC. Candidate gene approaches as well as genome-wide array-based studies have identified several genes with aberrant promoter DNA methylation in BE and EAC, and in many cases the epigenetic alterations that were found in EAC were also seen in BE.

In general, both genetic and epigenetic abnormalities are seen in BE before the development of dysplasia or EAC. This has important implications if these molecular alterations are to be used as assays to predict the risk of BE progression, since while it may be true that certain tumor suppressor genes are inactivated in many cases of BE, most individuals with BE will not progress to dysplasia or cancer.

Given the limitations of histopathology, genomic and epigenomic analysis has the potential to improve the precision of risk stratification. Specific gene mutations, chromosomal instability, and genetic diversity are associated with neoplastic progression, and it is foreseeable that assays to detect these features could be used to support the pathological assessment of disease and to select patients for more intensive surveillance.

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Key Points:

1. Genetic and epigenetic alterations play a central role in the formation of Barretts esophagus and esophageal adenocarcinoma.
2. Global epigenetic alterations occur early in the Barretts esophagus to esophageal adenocarcinoma sequence.
3. Genomic analysis of esophageal adenocarcinoma and Barretts esophagus has revealed a set of commonly altered genes that are likely drivers of cancer formation in the esophagus.
4. There is considerable inter and intra-lesional genetic and epigenetic heterogeneity in Barretts esophagus and esophageal adenocarcinoma.
5. It is likely that there are multiple different evolutionary pathways that drive the molecular evolution of Baretts esophagus to esophageal adenocarcinoma.

Figure 1:
Representations of initiation and progression of BE to EAC showing clonal evolution secondary to genomic and epigenomic alterations.