Abstract

The multistage carcinogenesis of esophageal adenocarcinoma is a process of clonal evolution within Barrett’s esophagus neoplasms. The initiating event for Barrett’s esophagus is unknown, but is associated with chronic gastric reflux which probably also promotes progression. Inactivation of both alleles of CDKN2A appear to be early events causing clonal expansion. Clones with TP53 inactivated expand if they have already inactivated CDKN2A. After TP53 has been inactivated, tetraploid and aneuploid clones tend to develop. The final events that lead to invasion and metastasis are unknown. Evolutionary biology provides important tools to understand clonal evolution in progression and cancer prevention.

Keywords: Carcinogenesis; Evolution; Barrett’s esophagus; Cancer prevention

1. Introduction

In 1954, Armitage and Doll analyzed cancer incidence as a function of age and hypothesized that the generation of a malignant neoplasm required multiple mutations [1]. This hypothesis was later supported by the discovery of tumor suppressor genes [2,3] and oncogenes [4]. In 1976, Nowell argued that cancer evolves from natural selection amongst cells within a neoplasm [5]. Nowell’s clonal evolution hypothesis explains how neoplasms progress to cancer and why cancer is so difficult to cure. Today, the details of which genes are targeted during progression and how mutations in those genes affect clonal evolution are not fully understood for any human cancer, with the possible exception of Retinoblastoma [6]. Barrett’s esophagus (BE) provides one of the best models for studying human carcinogenesis in vivo. We will review the current state of knowledge of multistage carcinogenesis in Barrett’s esophagus [7–12] while identifying important gaps in our understanding and highlighting the hallmarks of cancer as they apply to this neoplasm [13]. The ultimate goal of understanding carcinogenesis is the prevention and cure of cancer. BE is a promising test case for cancer prevention because the effects of an intervention can be monitored genetically and the difficulties that must be overcome for cancer prevention in BE are likely to plague cancer prevention in all solid tumors.

1.1. Multistage carcinogenesis

Pre-malignant solid neoplasms are generally constrained by limited space and resources. Thus, any neoplastic clone that acquires a somatic mutation which results in an increase in its survival or reproductive potential will tend to spread in the neoplasm to the detriment of competitor clones and normal cells that lack the beneficial mutation. This is true even in an expanding neoplasm, but is exacerbated when space and nutrients limit an otherwise exponentially
expanding clone. Somatic mutations and epigenetic alterations provide variation in the population of neoplastic cells and natural selection filters that variation for lesions advantageous in clonal competition. Mutation rates often increase dramatically during progression due to the development of genetic and epigenetic instability [14].

Multistage carcinogenesis is often, though certainly not always [1,15–17], described as a deterministic and linear series of lesions [18,19]. However, the predictions of a pioneering linear model of colorectal carcinogenesis [18] were recently tested and shown to be supported in only 6.6% of tumors [20]. The evolutionary theory of carcinogenesis predicts that progression will be characterized by intermittent selective sweeps of clones with advantageous mutations, often called clonal expansions. Between these homogenizing sweeps, and perhaps even during the sweeps, genomic instability will tend to re-establish genetic diversity within the neoplasm. Characterization of a stage of carcinogenesis by the simple presence of a mutation obscures the genetic diversity that both drives progression and accounts for therapeutic resistance. Such a characterization may be misleading in practice because the mutation may be driven extinct, clonally expand or remain a focal lesion depending on the competitive interactions of the different clones within the neoplasm. Mutations that do not confer a selective advantage on a clone, such as a mutator mutation [21] or the loss of the first allele of a tumor suppressor gene that is not haploinsufficient, will not sweep through the population of neoplastic cells and so may not define a distinct stage. Such a mutation is much more likely to go extinct than come to dominate the neoplasm [22]. A strict sequence of lesions described by a linear model requires that mutations late in progression are only selectivity advantageous for a neoplastic clone, or are otherwise more likely to occur, in the presence of all the previous mutations. This assumption remains untested in most descriptions of multistage carcinogenesis. Furthermore, many studies have demonstrated the presence of many more mutations in cancer cells than are described in linear models, leading to the identification of genetic instability in neoplastic progression and the phenomenon of genetic hitchhikers [14,23,24]. For all of these reasons, diagrams of asexual

Neoplastic Progression in Barrett Esophagus

![Diagram](image)

Fig. 1. An example of neoplastic progression in Barrett’s esophagus. Frequency in the neoplasm is shown on the Y-axis and time is on the X-axis. Clonal expansion along the Y-axis (frequency) with time represents clonal expansion in the two-dimensional surface of the BE epithelium. Clones evolve within a neoplasm through processes of mutation, natural selection and genetic drift that appears to take decades [28,29,33,111,129]. Evolutionarily neutral mutations will randomly increase and decrease in frequency unless they occur in a cell that also has a selectively advantageous mutation. In this case they are called ‘hitchhikers.’ Hitchhikers may occur before (hitchhiker 1) or after (hitchhikers 2 and 3) the occurrence of the advantageous mutation, as long as they arise before the selective sweep is complete. Loss of each allele of CDKN2A (p16) provides a selective advantage to the mutant clone and drives a selective sweep of that clone through the neoplasm [23]. Lesions in TP53 appear to only expand in the background of a CDKN2A lesion and are thought to be associated with chromosomal instability. Aneuploid clones typically arise within TP53- clones [16] and esophageal adenocarcinomas are usually aneuploid [100–102]. Adapted and reprinted with permission from [32].
evolution, introduced by Crow and Kimura [25] and shown in Fig. 1 for BE, may reflect the salient processes in carcinogenesis more realistically than descriptions that do not consider the competitive aspects of clonal evolution. These evolutionary models make testable predictions of the relative fitness values and competition between clones in neoplasms.

1.2. Barrett’s esophagus

Barrett’s esophagus is the only known precursor to esophageal adenocarcinoma (EA). It is defined as the presence of intestinalized metaplasia in the esophagus. In BE, a single layer of columnar cells replaces the normal stratified squamous cells of the esophagus and forms crypts reminiscent of intestinal crypts except that they excrete mucins rather than absorb nutrients [26]. BE is associated with chronic gastroesophageal reflux and approximately 10% of patients with gastroesophageal reflux disease have BE at endoscopy [7]. Patients with BE are at least 30-fold more likely to develop EA than patients without BE [27] though only 0.5–1% progress to EA per year [28,29]. Thus, large cohorts must be followed for many years in order to observe the development of enough adenocarcinomas to provide statistical power for most studies.

There are three clinical factors that make BE an excellent model for studying progression in solid tumors. First, unlike many other benign tumors such as an adenomatous polyp in the colon, BE is not surgically removed upon detection. Esophagectomies have 8% morbidity and mortality in high volume hospitals and 23% morbidity and mortality in low volume hospitals where the vast majority of operations are performed [30]. Thus, current recommendations are for serial endoscopic biopsies for the early detection of cancer [31]. Second, BE is easily visualized by endoscope and so can be targeted for biopsy sampling. Third, it can be safely and painlessly biopsied using standardized protocols to track the progression of the disease over time. Furthermore, the genetic lesions involved in BE, such as loss of p53, CDKN2A and the evolution of aneuploidy, are common to many solid tumors.

Nowell’s hypothesis for the role of clonal evolution in progression has been supported by a number of studies in BE [5]. Natural selection in BE [32] is probably driven by competition for space within the epithelial layer of the BE segment, rather than resource limitations. The size of a BE segment generally remains stable over time [33] while mutant clones expand and drive other clones extinct within the segment [23]. Furthermore, measures of clonal diversity, based on species diversity measures from ecology and evolutionary theory, have been shown to be useful in predicting progression to EA [34].

2. Initiation

Initiation of BE remains a mystery [32,33,35,36] in part due to the practical difficulties of following a large cohort of GERD patients to observe the transition to BE. Another high risk cohort that might be used to illuminate BE initiation is the set of patients that have undergone esophagectomy which removed all previous BE epithelium. BE often recurs in these patients and prevalence of BE increases with time [37,38].

It is clear that BE is associated with gastroesophageal reflux [26], so BE may just be the result of altered differentiation of esophageal epithelium due to the reflux environment. A comparison of gene expression levels between BE, esophageal squamous, gastric and duodenal mucosae identified 38 genes specifically up-regulated in BE [39]. Attention has focused on the homeobox genes CDX1 and CDX2, neither of which is expressed in normal squamous esophageal epithelium or normal gastric epithelium [40,41]. However, CDX2 is expressed in esophagitis, prior to the detection of BE and is found in virtually all BE samples [40,41]. CDX1 expression only occurs in the BE samples [40,41], though not in all BE samples [40], and is associated with demethylation of its promoter [41]. The importance of CDX2 in the transition to BE is supported by a transgenic mouse model in which expression of CDX2 in the stomach of the mouse generated intestinal metaplasia [42].

The columnar epithelium of BE and proximity to the stomach has led to the suggestion that BE may be established by gastric cardia migrating up into the esophagus. This hypothesis is not supported by experiments in dogs that induced BE while leaving a barrier of squamous epithelium between the gastric cardia and the BE [43]. Alternatively, BE may be established by colonization from esophageal gland ducts, a hypothesis supported by other experiments on dogs [44,45].

Finally, BE may be caused by a mutation that confers a competitive advantage on the mutant clone in the abnormal reflux environment. The best candidate mutation for this last hypothesis is loss of the CDKN2A tumor suppressor gene. Since, CDKN2A alterations are found in over 85% of BE patients at the first endoscopy when the BE epithelium is assayed [46], and clones with CDKN2A alterations often fill the entire Barrett’s
segment [23], the reflux environment may directly select for cells that have lost CDKN2A expression. Evidence against this hypothesis includes the minority of BE patients that show no CDKN2A alterations by LOH, mutation or hyper-methylation as well as the larger group of patients that have at least one biopsy without those alterations. Yet these exceptions may be explained by alterations in other loci of the CDKN2A pathway including Rb and Cyclin D1.

3. Progression

The gold standard for measuring the importance of an event in progression is its effect on the probability of developing cancer in prospective studies. Only a few lesions have been so rigorously tested and even those have not been validated in independent cohorts.

3.1. Reflux and wound healing

While gastroesophageal reflux appears to play a central role in initiation of BE, its role in progression is less clear. The refluxate is a complex combination of gastric acids and bile salts [47]. In cell culture experiments, both acid pulses [48,49] and bile salts [50] have been shown to increase proliferation. Daily cycles of reflux are likely associated with wounding of the Barrett’s epithelium and so may trigger wound healing responses which provide some of the hallmarks of cancer [13] including mitogens, release of growth inhibition and suppression of apoptosis in the base of the crypts [51,52]. This would explain the observations of over-expression in a variety of oncogenes [53–62] along with the scarcity of oncogenic mutations [7,63–66]. Inflammation is a common observation in BE and may increase both proliferation and mutagenesis [67]. Repeated wounding and repopulation cycles should also select for mutant clones with reproduction and survival advantages [68]. However, such wounding is difficult to study in vivo in humans except in the extreme condition of erosive esophagitis, which is rare in adequately treated BE patients, most of whom are medicated with acid-suppressive proton pump inhibitors (PPIs) [69].

3.2. CDKN2A

The most common genetic (and epigenetic) alteration in BE is inactivation of CDKN2A on chromosome 9p. As was noted above, LOH, promoter hypermethylation, or sequence mutations have been observed in over 85% of BE patients, even at the earliest stages of progression [46,70–74]. The fact that the same sequence mutation, or pattern of LOH in loci on chromosome 9p, can be observed across centimeters of BE tissue shows that CDKN2A inactivation is associated with selective sweeps [23] and so breaches the tumor suppressive mechanism of crypt structured tissue architecture [75]. Most mutations should either be flushed from the crypt, if they occur outside of the tissue stem cell pool, or remain isolated as a small population of mutant stem cells in the bottom of single crypt, but loss of CDKN2A leads to the spread of the mutant clone across hundreds of thousands of crypts. It is unknown if this clonal expansion occurs through wound healing or through competition between crypts driven by crypt death and bifurcation.

3.3. TP53

Loss of TP53 is one of the most common events in neoplastic progression [76] and is generally associated with loss of growth inhibition from a variety of cell stresses [77]. In BE it seems to only occur in the genetic background of a previous loss of CDKN2A [23]. The reason for this ordering of events is unknown but may be explained if loss of CDKN2A is necessary for clonal expansion. In this hypothesis, TP53 might be lost before CDKN2A, but would be isolated as a virtually undetectable mutant stem cell population at the bottom of a single crypt until the clone also lost CDKN2A and expanded to a clinically detectable size. The frequency of TP53 lesions may then expand either as a hitchhiker on a CDKN2A selective sweep, or due to the selective effects of loss of TP53 itself within a population of cells that have already lost CDKN2A and breached the crypt barrier to clonal expansion.

Loss of TP53 may occur through either LOH or sequence mutations. Loss of TP53 is an important event in BE progression [78–81] because patients with LOH in TP53 are 16 times more likely to progress to EA than patients without TP53 LOH [82]. Furthermore, the larger the clone with TP53 LOH, the more likely the patient is to progress to cancer [83]. Both sequence mutations and LOH in TP53 appear to provide a competitive advantage to the clone [23,84]. Loss of TP53 probably has at least three roles in progression by suppressing apoptosis and preventing cell cycle arrest [85], potential sources of competitive advantage for a clone, and permitting genetic instability which may increase the generation of viable genetic variants [86–88].
3.4. Tetraploidy

Tetraploidy, defined as an increased 4N DNA content, and has been observed in 15% of BE patients in one large cohort [89]. A threshold of 6% 4N fraction by flow cytometry was derived from a receiver operating characteristic (ROC) curve which determines the threshold that maximizes both sensitivity and specificity for future progression to cancer [90]. Patients with >6% 4N fraction were 11.7-fold more likely to progress to EA than patients without a tetraploid population, showing that this is an important event in BE progression [90]. Patients with increased 4N fractions were also more likely to develop aneuploid clones [91]. By doubling the genome, a cell can lose up to three alleles while still retaining gene function and so may be more robust than a diploid cell to genomic instability and the chromosomal losses that generate an aneuploid clone.

3.5. Aneuploidy

The detection of aneuploidy by flow cytometry is associated with a 9.5-fold risk of progression to cancer [90,92]. Aneuploidy typically appears after TP53 alterations have developed [16]. One hypothesis for integrating the above observations is that loss of TP53 is permissive for both the development of tetraploidy and subsequent chromosomal instability. The TP53-tetraploid clones may then lose pieces or entire chromosomes to generate aneuploid clones. It is unknown if the generation of a viable aneuploid or tetraploid clone depends on further genetic lesions beyond the inactivation of TP53. It is known that the larger the clone with aneuploidy, tetraploidy or p53 LOH, the more likely the patient is to progress to EA [83]. Presumably, a large field of genetically unstable cells is more likely to produce the mutations necessary for carcinogenesis than a small field.

3.6. Telomerase

Because senescence via the erosion of telomeres is a potential cancer suppressive mechanism, activation of telomerase or the ALT pathway is a common event in neoplastic progression [13]. There is conflicting evidence for relative telomerase activity between squamous and Barrett’s esophagus epithelium [93,94]. However, EA appears to have high levels of telomerase expression compared to either BE or squamous epithelium [93,95]. It is unknown if there are any regularities in the timing of telomerase activation during BE progression or if it is equally likely to be activated at any point during progression. The role of telomerase in BE progression remains an important topic for future research.

3.7. Neo-angiogenesis

Because the Barrett’s epithelium is a single layer of cells on the surface of the esophagus, it is well enervated by capillaries and does not suffer the adverse effects of hypoxia. Thus, there should be no hypoxic selection for neo-angiogenesis until a three-dimensional mass forms. However, even in a two-dimensional surface of BE epithelium, stimulation of neo-angiogenesis might still give a reproductive advantage to a clone by increasing the supply of nutrients. Neo-angiogenesis is common in EA [96–98] and VEGF levels are higher in EA than in BE [99]. Angiogenesis in BE has mainly been studied in surgically resected specimens from patients with EA or high grade dysplasia [97,98], and given the diffusion of angiogenic signals, may not be representative of a pre-malignant neoplasm. It is unknown when neo-angiogenesis occurs relative to the other steps in BE progression.

3.8. Invasion and metastasis

Most EAs are aneuploid [100–102], yet aneuploidy arises before cancer [90]. We are therefore missing the lesions that transform a pre-malignant aneuploid clone into a malignant clone. Disruption of E-cadherin can lead to invasion and metastasis [103]. E-cadherin expression appears to decrease with progression from squamous to BE to EA [104,105] though there is little evidence for disruption of the gene itself [70,106]. Alterations in APC, on chromosome 5q, have been reported in some cases preceding malignancy [16,107] and in other cases following the development of EA [16], obscuring its role in EA carcinogenesis. In all cases tested, TP53 LOH preceded LOH in 5q, suggesting that if APC LOH does play a role in carcinogenesis, it acts relatively late in progression [16].

3.9. Multistage carcinogenesis in BE

I have argued that strict linear models of multistage carcinogenesis may be misleading because they suggest genetic dependencies between the stages that usually have not been tested and obscure the genetic heterogeneity that drives neoplastic progression. A recent study has shown that the degree of genetic
heterogeneity within a BE segment predicts progression to cancer [34]. Some genetic dependencies have been shown in BE. There is evidence that CDKN2A lesions precede TP53 lesions in the vast majority of cases [23]. Furthermore, there is evidence that both TP53 lesions and tetraploidy predispose to the development of aneuploidy [16,91]. The relative ordering of TP53 loss and tetraploidy has not been determined. Cell stresses and DNA damage may cause increased activation of TP53 as a G2 checkpoint and thus select for TP53 lesions, or TP53 lesions may be permissive for tetraploid cells that cycle through an octoploid G2 phase. It is unclear if there are genetic dependencies that would place telomerase activation and neo-angiogenesis into this sequence of events.

### 3.10. Clinical implications

BE is one of the few neoplasms in which biomarkers for risk stratification have been evaluated in phase IV biomarker (prospective cohort) studies [108]. Both TP53 LOH and ploidy lesions are promising biomarkers [82,90]. The importance of ploidy lesions for risk stratification has been validated in a second cohort [92] and is now being used to adjust frequency of surveillance in clinical practice. However, TP53 LOH has not yet been validated for cancer outcome in a second cohort, and so probably should not be used for patient management until its importance can be confirmed. CDKN2A lesions are so common in BE that they are unlikely to be useful for risk stratification when considered alone. However, even the best single biomarker is likely to be limited by trade-offs between sensitivity and specificity. This problem may be ameliorated by the use of panels of biomarkers, which may include CDKN2A as one component of risk stratification.

The evolution of clones throughout BE progression implies that biomarker status will change over time and continued monitoring will likely be necessary at intervals appropriate to the severity of risk.

### 4. Cancer prevention

At first glance, BE appears to be an ideal case for cancer prevention. Patients with BE are a high risk population, key genetic events in progression are known, and the only alternative intervention is esophagectomy, with high mortality and morbidity. Furthermore, response to therapy can be tracked endoscopically, histologically and genetically.

A recent, multi-center, randomized, phase III clinical trial suggests that careful ablation of the BE epithelium by photodynamic therapy (PDT) may provide an effective cancer prevention intervention [109]. A total of 208 BE patients with HGD were randomized to PDT with acid suppression by omeprazole (N=138) versus omeprazole alone (N=70). PDT resulted in the primary outcome of more frequent regression of HGD (77 vs. 39%, \( P<0.0001 \)). More importantly, the secondary outcome of cancer was observed in half as many PDT treated patients (13%) compared to control (28%, \( P=0.006 \)). However, average follow-up was a little less than a year (maximum 3.6 years), so the long term effects of PDT remain an open question.

There is observational evidence that non-steroidal anti-inflammatory drugs (NSAIDs) prevent progression to EA. A meta-analysis of two cohort studies and seven case-control studies (totaling 1831 cases of EA) found a protective effect of NSAIDs (odds ratio = 0.57, 95% confidence interval [CI]: 0.47–0.71) [110]. A recent cohort study that accounted for use of NSAIDs throughout follow-up found that NSAIDs reduced the risk of progression to EA by a factor of 5 (hazard ratio = 0.20, 95% CI: 0.10–0.41) [111]. These results are promising and make a strong case for randomized, placebo controlled trials to test if NSAIDs can be used as cancer prevention agents in BE [112]. The prevalence of inflammation in BE and the efficacy of NSAIDs in cancer prevention has led to interest in cyclooxygenase-2 (COX-2), an inducible inflammation response gene modulated by NSAIDs. COX-2 is over-expressed in BE [113–115] suggesting that COX-2 inhibitors might act to prevent EA. However, recent studies found that COX-2 inhibitors have significant cardiovascular toxicity [116–118] and this has drained enthusiasm for their use in low-risk populations.

BE neoplasms are genetically heterogeneous [16], which raises the specter of resistance to potential interventions. In fact, there is already some evidence for resistance to PDT, an alternative to surgical resection in which the BE epithelium is exposed to a photosensitizing agent and then ablated light exposure. Some TP53 mutant clones appear to be resistant to PDT [119–122]. Another form of resistance results in the apparent regrowth of squamous epithelium during PPI treatment [69,123] except that BE epithelium sometimes persists underneath the squamous epithelium [69]. This is clinically problematic for both cancer prevention and surveillance because the BE can no longer be visualized endoscopically and late stage adenocarcinomas may develop before they can be
It seems likely that future therapies that target genetic alterations in BE progression will also have to overcome their inevitable selection for resistant mutants [125]. It remains to be seen if the AspECT chemoprevention trial using PPIs and aspirin [112] or the CBET trail using the COX2 inhibitor celecoxib [126] will show evidence of resistance. Unfortunately, the patients that are least likely to have resistant neoplasms, at the earliest stages of progression, are at the lowest risk for progressing to cancer. Thus, the risk of complications from an intervention in early stage patients are more likely to out-weigh the benefits of cancer prevention effects compared to high-risk patients. These concerns apply to virtually all neoplasms and are major challenges to the field of cancer prevention.

5. Conclusions

BE neoplasms may be genetically surveyed in both space and time. This has allowed for the rigorous determination of lesions involved in BE progression, primarily inactivation of CDKN2A and TP53 as well as chromosomal instability. Although, we have learned a lot about carcinogenesis in BE, future work will require prospective studies with multi-institutional collaborations to collect enough cancers for statistical power to measure the roles of multiple genetic lesions and their interactions. There are many important questions that remain unanswered: what triggers the initiation of BE? How do clones spread laterally in the BE epithelium? What are the roles of telomeres and telomerase in BE progression? Are there further genetic events that lead to aneuploidy once TP53 has been inactivated? What loci are targeted by chromosomal instability in aneuploid neoplasms that cause invasion?

The successes in unraveling the genetic lesions in BE could be replicated in other neoplasms as long as multiple samples are taken from a pre-malignant neoplasm, allowing for clonal ordering by genetic dependencies [127,128], the epithelial cells can be adequately purified from the contaminating stromal cells, and patients can be followed prospectively to a cancer end-point. Furthermore, since neoplastic progression is a process of clonal evolution, understanding that evolution will require the measurement of allele frequencies in the neoplastic cell populations [23,83]. The evolutionary and ecological dynamics that drive progression and response to therapy remain understudied areas of great importance. For this reason, cancer biology would greatly benefit from interdisciplinary training and collaborations with ecologists and evolutionary biologists.

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