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Review

Natural selection in neoplastic progression of Barrett's esophagus

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Abstract

Neoplasms progress to cancer through a process of natural selection. The rate of evolution, and thus progression is determined by three parameters: mutation rate, population size of the evolving neoplastic cells, and intensity of selection or rate of clonal expansion. All three parameters are reviewed in the context of Barrett's esophagus, a pre-malignant neoplasm. Although Barrett's esophagus is an ideal model for the study of neoplastic clonal evolution, similar studies may be carried out in a wide variety of human neoplasms. Evolutionary analyses provide insights for clinical management, including rates of progression to cancer and emergence of resistance to interventions.

Keywords: Neoplastic progression; Natural selection; Barrett's esophagus; Evolution; Cancer

Contents

1. Natural selection in neoplastic progression

A neoplasm is a microcosm of evolution. This fact lies at the heart of why we get cancer and why it has been so

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hard to cure. Within a neoplasm, mutant clones compete for space and resources [1–5]. Those that have a competitive advantage and are more aggressive will tend to spread in the neoplasm until a mutation produces an invasive phenotype and the neoplasm becomes malignant. Therapies add a new selective force to the neoplasm and tend to select for resistance [6–12]. Thus, natural selection leads to both progression and relapse.

There are three necessary and sufficient conditions for natural selection: (1) There must be variation in the population. (2) The variation must be heritable. (3) The variation must affect the number of offspring contributed to the population by an individual [13]. When these three conditions are met, natural selection ensues in the population, whether it be a population of organisms or cells in a neoplasm. Cells in neoplasms evolve by natural selection because: (1) There is genetic and epigenetic variation within a neoplasm due to somatic mutations and methylation [1–5]. (2) That variation is heritable because it is encoded in the nucleotides and methylation of the DNA. (3) The genetic and epigenetic alterations lead to the phenotypic changes embodied by the hallmarks of cancer, including liberation from a reliance on growth signals, insensitivity to anti-growth signals, evasion of the immune system, suppression of apoptosis, neoangiogenesis, and finally invasion and metastasis [14,15]. All of these phenotypes provide a competitive advantage to the mutant clone over competing clones that lack those phenotypes. Nowell recognized the importance of natural selection in neoplastic progression in 1976 [16]. However, only recently has the field of cancer biology progressed to the point of testing Nowell's hypothesis. Barrett's esophagus, a premalignant neoplasm of the esophagus, is one of the few neoplasms in which Nowell's hypothesis can be studied in detail, over time, in vivo. Here we review what is known about the parameters of clonal evolution in Barrett's esophagus along with the important open problems that remain.

2. Parameters of the rate of evolution

If neoplastic progression is an evolutionary process, then the rate of evolution should be associated with the rate of progression to cancer. Evolution is traditionally and most narrowly defined as changes in allele frequencies. There are three parameters that determine the rate of evolution in a neoplasm: (1) mutation rate, (2) population size, and (3) strength of selection. The faster that new mutations accumulate in a neoplasm, the faster the neoplasm will acquire all the necessary carcinogenic lesions for malignancy. This is a function of both the mutation rate per cell and the number of cells in the neoplasm. The larger the population of neoplastic cells, the more likely that at least one cell will acquire a carcinogenic mutation. The strength of selection is important because the faster a mutant clone spreads in the neoplasm, the larger the population of mutant cells available to acquire further carcinogenic mutations. If clones grow slowly, then it is unlikely that a single cell will acquire all the necessary and sufficient mutations to become malignant [17–19].

3. Barrett's esophagus as a model of clonal evolution in neoplastic progression

Nowell's hypothesis of clonal evolution in neoplastic progression should apply to all neoplasms. However, it has been difficult to study in most neoplasms because either the precursor lesions are not easily identified, or if they are identified, they are removed and so cannot be studied longitudinally to see how the clones evolve. Barrett's esophagus (BE) is an exception.

BE is a pre-malignant neoplastic [20] condition of the esophagus, that is the only known precursor of esophageal adenocarcinoma (EA) [21,22]. BE is defined by the presence of crypt structured, intestinal metaplasia in the esophagus replacing the normal multi-layered squamous epithelium. BE develops as a complication of chronic gastroesophageal reflux disease (GERD) in approximately 5–12% of GERD patients [23,24]. The only proven therapy for BE/EA is an esophagectomy. Unfortunately, esophagectomies have a morbidity and mortality of 3–8% in high-volume hospitals and 16–23% in low-volume hospitals where most surgeries are performed [25–28]. Because only 0.5–1% of patients with BE progress to EA annually [29,30], periodic endoscopic biopsy surveillance using a systematic biopsy protocol is recommended for early detection of cancer [21]. Thus, the clinical standard of care makes it possible to study clonal evolution of BE in vivo, longitudinally in a cohort of patients.

BE is an ideal model for the study of neoplastic progression not only because it can be tracked longitudinally, but also because it exhibits some of the most common genetic lesions in human solid tumors. Loss of p16 (CDKN2A/INK4A) occurs early in BE progression [31,32]. Loss of p53 (TP53) occurs after loss of p16 in almost all cases [20]. Eventually tetraploidy (increased 4N fractions) and aneuploidy develop before cancer [33].

We will review the three parameters for the rate of evolution in BE. A variety of forms of genetic instability have been measured in BE and likely play a role in progression. Population sizes have been measured both in terms of segment length and clone sizes. Evidence for natural selection comes from both studies of the frequencies of lesions across neoplasms and mutation frequencies within neoplasms.

4. Mutation rate

Mutation rates are difficult to study in humans in vivo. Most estimates of mutation rates either come from cell culture [34,35] or the big blue mouse [36]. However, there is no doubt that BE neoplastic cells have higher mutation rates than normal tissue because a variety of genetic and epigenetic

lesions have been observed in BE at higher frequencies than are observed in normal tissues.

Allelotype studies, using a sparse set of microsatellite markers scattered across the genome, have found evidence of loss of heterozygosity (LOH) in BE on virtually every chromosomal arm of the genome [37,38]. These results cannot distinguish between physical loss or deletion of a chromosome arm, leaving only one allele at the microsatellite locus, from gene conversion or mitotic recombination, leaving two identical copies of an allele at the microsatellite locus. However, assays that measure copy number changes, such as fluorescent in situ hybridization (FISH) and comparative genomic hybridization (CGH), can distinguish physical loss from gene conversion, though they cannot distinguish wildtype loci from LOH due to gene conversion. FISH and CGH studies have found both gains and losses of chromosomes in Barrett's epithelium [39–43]. LOH without copy number change, for example, by gene conversion or mitotic recombination, has been observed in retinoblastoma [44], breast cancer [45], colon adenomas [46,47], bladder cancer [48] and leukemia [49], as well as in Barrett's esophagus, recently (Wongsurawat et al unpublished observations).

Chromosomal instability is only one form of genetic instability observed in BE. Changes in microsatellite allele sizes have also been commonly observed [20,50], though not at a frequency to qualify as microsatellite instable [51,52]. These new alleles, or "microsatellite shifts," are often observed in tetranucleotide repeats [52], rather than the dinucleotide repeats used to define microsatellite instability in colorectal cancer [51]. The cause of these microsatellite shifts is unknown.

Sequence mutations have been detected in p16 [32], p53 [53] and K-ras, though K-ras mutations have only been detected late in progression[54,55]. Inactivation of p16 by sequence mutations occurs in 15% of BE patients and often does not affect the alternative reading frame of ARF [32]. A much more common form of inactivation of p16 is due to hyper-methylation of the p16 promoter, which is detected in approximately 61% of BE patients and p16 LOH detected in 57% of Barrett's patients [32]. APC has also be reported to be frequently methylated in BE [56,57].

5. Population size

The size of a population is a fundamental constraint on the rate at which it can accumulate mutations and evolve. Until the mid 1970s, there were reports of BE segments growing over time [58–60]. However, since that time, such reports have virtually disappeared [61]. It may not be a coincidence that H2 blockers, the first effective acid suppression medications, were introduced in the mid 1970s. Today, in the vast majority of cases, the length of the Barrett's segment remains stable over time [61], though sometimes it partially regresses with the re-growth of squamous tissues [62,63]. This observation leads to a striking conclusion: the initiation and establishment of a BE segment must occur quickly relative to physicians ability to detect it so that it is virtually impossible to catch BE in the act of establishment.

If the rate of evolution depends on population size, then a logical prediction of the evolution of neoplasms is that patients with larger pre-malignant neoplasms should be more likely to progress to cancer than patients with smaller neoplasms. The evidence in BE is equivocal. In two retrospective, case-control studies, patients that had progressed to cancer tended to have had larger BE segments than the controls [64,65]. The one prospective cohort study that examined this issue found a trend for longer BE segments to progress to cancer, but the trend was not statistically significant [66]. However, closer inspection of this cohort showed significant associations between the sizes of clones with p53 LOH, aneuploidy or tetraploidy and progression to EA [67]. Thus, the size of the genetically unstable cell population was a strong predictor of neoplastic progression.

6. Strength of selection

The strength of selection on a particular allele is best measured by the rate at which it spreads through a population. This has been difficult to measure in most neoplasms for two reasons: very few neoplasms can be tracked over time and few investigators have measured the frequency of alleles within a neoplasm by assaying multiple biopsies [20,68].

The close association between gastroesophageal reflux and the etiology of BE suggests that under the abnormal environment of reflux, BE cells have a competitive advantage over squamous cells in the esophagus. BE is defined in part by the presence of goblet and other mucus-secreting cells that probably help to protect the Barrett's epithelium from the acids and bile salts in the reflux [69].

6.1. Natural selection on specific loci

A cross-sectional analysis that analyzed the frequency with which different genetic lesions went to fixation (>90% of the neoplasm) found strong evidence that loss of p16, either by LOH, mutation or methylation, conferred a selective advantage on the mutant clone [20]. This corroborates evidence from the high frequency with which p16 is lost in BE [32,70–72].

Loss of p53 was not observed in epithelium that had not also lost p16 [20] suggesting that either loss of p53 does not give a competitive advantage to a p16 wildtype clone or that the crypt architecture of BE prevents a clone that has lost p53 from expanding beyond the crypt until it has also lost p16. Within a background of BE that had lost p16, clones with p53 lesions tended to go to fixation more frequently than would be expected by chance, giving further evidence that p53 LOH and sequence mutations are selectively advantageous in BE [20].

A large number of genetic losses have been observed in BE but have not yet been associated with clones that regularly grow to fixation. The strongest evidence is for aneuploidy and tetraploidy, which, though they do not tend to grow to fixation, are associated with significant risks of progressing to cancer [73]. LOH on 5q, 18q and 13q and loss of the Y chromosome are all commonly observed [37,38]. Losses on 5q are thought to target the APC gene [74] and losses on 13q may target the Rb gene, since it is in the p16 pathway. It is unclear what gene, if any, is being targeted on 18q because a large number of cancer-related genes can be found there, including SMAD4, DCC, and Bcl2. Cyclin D1 is also frequently overexpressed in BE [75,76].

6.2. Hitchhikers

The mere observation of a genetic or epigenetic alteration in a neoplasm is not sufficient evidence to establish its importance in neoplastic progression, even if the alteration is observed at fixation throughout the neoplasm or across multiple neoplasms. This is because an evolutionarily neutral alteration may spread in a neoplasm due to the fact that it occurs in a clone that has a selectively advantageous alteration elsewhere in the genome. This phenomenon is called hitchhiking in evolutionary biology and it has the potential to mislead entire fields within cancer biology. Evidence that an alteration is selectively advantageous must come from the consistent association of that alteration with clonal expansion [20], and may be contrasted with alterations that are almost assuredly evolutionarily neutral, such as microsatellite shifts in non-coding regions of the genome. This distinction between selective and hitchhiking alterations requires the study of enough neoplasms to detect the difference between the random occurrence of an alteration due to genetic or epigenetic instability and clonal expansions driven by natural selection.

6.3. Resistance

Interventions in cancer therapy and prevention, by definition, change the environment of the neoplasm and thus change the selective pressures on the neoplastic cells. The fact that these pressures select for clones resistant to the intervention is the single most important reason that cancer has proven so difficult to cure. Esophageal adenocarcinoma is no exception. Both chemotherapy and radiotherapy have proven ineffective in this disease [77,78]. The only proven cure for esophageal adenocarcinoma is esophagectomy, which can be successful for early cancer with localized disease [79–82].

There has been recent interest in photodynamic therapy (PDT) in which lasers, in combination with light reactive dyes, are used to burn away the BE epithelia in order to prevent progression to EA [83–86]. However, there are now reports that patches of BE that have lost p53 tend to survive PDT and these may explain reported cases that progress to EA after PDT [87,88].

7. Open problems

Neoplastic evolution is probably better understood in BE than any other human solid neoplasm, and is summarized in Fig. 1. However, there are many unsolved mysteries that require further study.

7.1. Initiation of Barrett's esophagus

It is unknown what triggers the transition from squamous to Barrett's epithelium in GERD patients. Competing hypotheses [89,90] include: (1) A change of expression in the esophageal epithelium due to the abnormal exposures of gastric reflux. (2) Migration of gastric cardia up into the esophagus with subsequent changes in differentiation to produce goblet cells. (3) Colonization of cells from esophageal gland ducts, and (4) A genetic or epigenetic lesion in a cell that produces the BE phenotype and provides a competitive advantage such that the Barrett's clone expands to displace the normal squamous epithelium. The best candidate locus for this last hypothesis is the p16 tumor suppressor gene. Since p16 alterations are found in over 85% of BE patients at the first endoscopy when the BE epithelium is assayed, and clones with p16 alternations often fill the entire Barrett's segment, loss of p16 in a reflux environment of the esophagus may cause BE. Evidence against this hypothesis includes the minority of BE patients that show no p16 alterations by LOH, mutation or 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 p16 pathway including Rb and Cyclin D1.

7.2. Genetic and epigenetic instability

Once a Barrett's neoplasm is initiated, the BE cells soon begin to collect genetic and epigenetic alterations. The cause of genetic and epigenetic instability is generally unknown, particularly early in progression. Although most BE patients are on powerful PPIs, many patients continue to reflux both gastric acids as well as bile [91]. Both acids and bile salts may be mutagenic, and so directly cause genetic instability, but they also may increase genetic instability by increasing cell turnover. Most biopsies of BE tissues show evidence of inflammation which may also cause genetic instability through the production of mutagenic oxygen radicals or through increased cell turnover. Finally, it may be the case that epigenetic silencing of mismatch repair enzymes, such as MLH1, or other genes involved in maintenance of genetic stability leads to increased genetic instability, though this has yet to be shown.

The different forms of instability, including methylation, microsatellite shifts, mitotic recombination, and chromosomal losses and gains, probably have different but interrelated etiologies. They are likely to have different rates of mutation and emerge or change at different times during progression. For example, loss of p53 is permissive for a variety

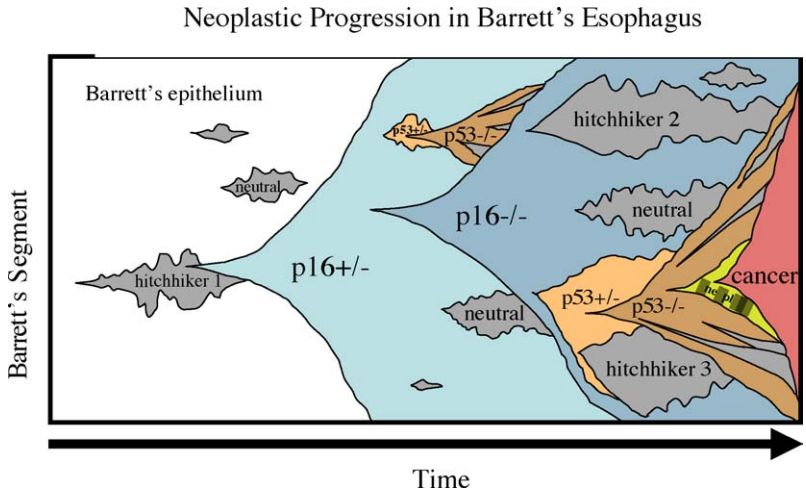

Fig. 1. Clonal evolution in Barrett's esophageal neoplastic progression. The *Y*-axis represents the vertical extent of a Barrett's segment and the *X*-axis shows change over time. Loss of each allele of p16 appears to give the clone a competitive advantage and leads to clonal expansion. p16 inactivation is either the initiating event for Barrett's esophagus or is an early event in progression. Lesions in evolutionary neutral loci probably occur in many clones within the neoplasms but whether or not they expand over time is a random process of genetic drift, unless they hitchhike on the clonal expansion of a selectively advantageous lesion. Hitchhikers may precede the selective mutation (hitchhiker 1 expands with the p16+/− clone) or arise during a selective sweep (hitchhikers 2 and 3). Expansion of a p53 mutant clone seems to require inactivation of p16, and so only grows large enough to be detected when it arises as a subsequent event. It is unclear if a p53 hemizygous (+/−) clone is evolutionarily neutral, and must expand as a hitchhiker as shown here, or has a competitive advantage over a p53+/+ clone. Loss of both p53 alleles probably increases genomic instability leading to diversification within the neoplasm and is permissive for the subsequent generation of tetraploid and aneuploid clones. Eventually, esophageal adenocarcinoma may evolve, typically deriving from an aneuploid clone. A mutant cell within a clone carries all the genetic lesions of the ancestral clone. In this example, the cancer would be a p16−/−, p53−/− aneuploid clone with at least one neutral hitchhiking lesion (hitchhiker 1).

of genomic alterations, and so the mutation rates are likely to increase after p53 loss.

7.3. Effective population size

No one has been able to identify the stem cell population in a BE crypt. Is it composed of just a few cells at the bottom of the crypt, similar to a small intestinal crypt in the mouse [92], or is the entire proliferating compartment evolving over time? Another possibility is that the number of crypts in a Barrett's segment, and thus the effective population size is changing over time, even though the segment length remains constant. Studies are on-going to address these questions.

7.4. Clonal expansion

In 1975, Cairns showed that the crypt architecture itself acts as a tumor suppressor because mutations that occur in most of the crypt will be purged from the crypt as the cells migrate up the crypt and slough off into the lumen [93]. Even if a mutation occurs in a stem cell of the crypt, it has no obvious way to colonize neighboring crypts. However, given that we see mutant clones covering more than 10 cm of a BE segment and hundreds of thousands of crypts, this barrier to clonal expansion has been breached. We do not know how.

Little is known about the dynamics of tissue architectures, such as crypt structured epithelium. What causes a crypt to bifurcate? How often do crypts die? If there is turnover in the population of crypts, then a mutation that causes a crypt to divide more frequently than other crypts or increase the chance that a crypt survives in the BE environment will tend to spread in the BE epithelium. This may be driven by a constant background rate of crypt death and bifurcation, or it may be driven by wounding caused by acid and bile reflux, and subsequent competition in the surviving epithelium to repopulate the denuded areas. Furthermore, wound healing may be accomplished by either crypt bifurcation or epithelial restitution in which a flat sheet of epithelium migrates to cover the wound and later invaginates to reconstitute the BE crypts. The implications of these different tissue architecture dynamics for neoplastic progression remain unknown.

7.5. Molecular alterations in progression

The best evidence for molecular alterations in BE progression involve lesions in p16, p53 and the generation of tetraploidy and aneuploidy [32,73,94]. However, there are likely other necessary alterations before BE epithelium may become malignant.

Genetic dependency ("clonal ordering") studies have been used to associate loss of chromosome arms, which may carry tumor suppressor genes with the development of EA [95,96]. This is how p16 and p53 were originally identified as important alterations that precede the evolution of EA and so may be necessary alterations for EA. While loss of 5 q and methylation of APC are common, they can occur both before and after the emergence of EA, suggesting that loss of APC is not necessary for EA, though it may affect prognosis [56]. Loss

of 18 q is commonly observed before EA, but as discussed in Section 6.1, the gene or genes being targeted by these losses is unknown.

Discovery of relevant oncogenes in progression is more difficult because they are often activated by point mutations, and so require extensive sequencing to be detected. In the future, high-density CGH and SNP chips may be able to pinpoint genes that are commonly amplified in BE. Studies of the common oncogenes, such as *myc* and *ras*, have not found any consistent alterations in BE pre-malignant epithelium [54,55,97,98]. Alternatively, chronic wound healing and inflammation may be providing the proliferative signal usually supplied by oncogenes during progression, and so there may be no relevant oncogenes in BE progression.

Aneuploidy is commonly observed late in progression but preceding EA. These aneuploid clones include large-scale chromosome gains and losses [42,99–101]. It is unknown what loci these gains and losses are targeting, and in particular, what alterations finally produce the invasive phenotype.

7.6. Insights for clinical management and computational models

An evolutionary understanding of neoplasms leads to two important insights. In order to solve the problem of cancer, we will either have to prevent cancer at a stage before somatic mutations are likely to have generated a resistant clone [102] or develop interventions that account for the clonal diversity within the neoplasm [103]. The problem with intervening early in progression is that only a minority of those patients would develop cancer in the absence of the intervention, so the costs of rare complications from the intervention may outweigh the benefits unless the intervention is very benign.

How can we develop therapies that avoid the evolution of resistant clones? We must find some way to interfere with clonal evolution. One useful initial step is the development of computational models that can represent the genetic heterogeneity within a neoplasm and the evolution of resistance [103]. Agent-based computational models can represent the genetic state of each cell in a neoplastic cell population along with stochastic neutral and selective mutations, apoptosis, limited space, resources and the competition that ensues [104]. These computational models act as pre-pre-clinical models that may be used as test-beds for exploring evolutionary strategies for cancer therapy and prevention. Two evolutionary strategies show promise in computational models: benign cell boosters and chemosensitive boosters [103]. A benign cell booster is an intervention that increases the fitness of the relatively benign cells in or around a neoplasm. Clonal competition results in driving the less benign cells extinct and thereby stalling progression (Fig. 2). Chemosensitive boosters are similar except it is the cells that will be sensitive to a chemotherapy whose fitness must be increased. In this case, the chemosensitive cells drive the resistant cells extinct so that when the chemotherapy is later applied, all

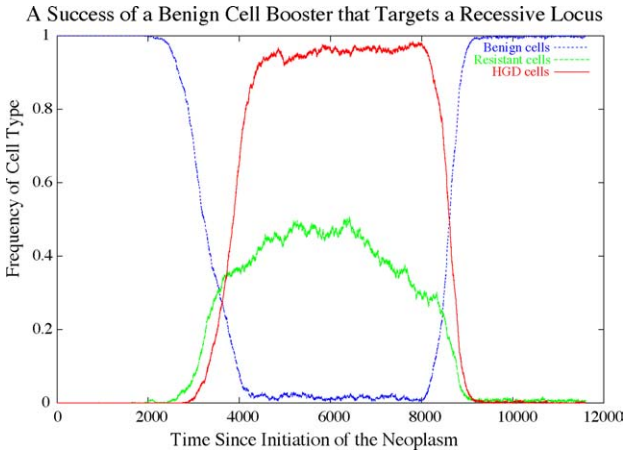

Fig. 2. An example run of a computational model simulating a benign cell booster applied to a pre-malignant neoplasm. Each time step represents half a day (8000 time steps ≈11 years). The benign cell booster, applied for 5 years starting at time step 8000, decreases the generation time of cells that still have at least one intact allele of a tumor suppressor gene, shown in blue. Resistance to the booster may evolve in the model (shown in green), but resistance is detrimental to the cell, since it prevents a benign cell from gaining the benefit of the booster. Cells that have progressed to high grade dysplasia (HGD, shown in red) have inactivated key tumor suppressor genes, including the one targeted by the benign cell booster. In the presence of the benign cell booster, benign cells out-compete HGD cells and prevent progression. Figure reproduced with permission from Maley et al. [103].

the cells in the neoplasm are vulnerable. We call this the "Sucker's gambit."

Both benign cell boosters and the Sucker's gambit remain only theoretical possibilities at the moment. How to instantiate them in biology remain open problems to be explored in pre-clinical models. There may well be better evolutionary strategies yet to be discovered for cancer therapy and prevention. It is intriguing to note that proton pump inhibitor (PPI) medications, which suppress gastric acids, may be acting as benign cell boosters by changing the esophageal environment and selective pressures such that squamous cells have a competitive advantage over BE cells. When PPIs are combined with some form of wounding the epithelium that fills the wound tends to be squamous [105]. However, there is also evidence of resistance to PPIs as benign cell boosters. In some cases, the squamous cells grow over the BE cells rather than replacing them [106]. This poses a clinical problem because the endoscopist can no longer see the BE epithelium, and so it may progress to EA undetected.

8. Conclusions

Barrett's esophagus has proven a fruitful model system of human neoplastic progression in which to test Nowell's hypothesis in vivo. All of the necessary components of natural selection in a neoplasm have been confirmed in BE including somatic variation, heritability of that variation, and differences in relative fitness of the clones due to that variation. The introduction of evolutionary theory to cancer biology

provides a useful set of quantitative analyses and explanations for neoplastic progression.

While BE may be an ideal model for exploring clonal evolution in neoplastic progression, it is by no means the only one. In order to study evolution, one must, at the least, be able to measure allele frequencies in the neoplasm. This requires the technology to assay genotypes in small numbers of cells. Measuring allele frequencies is most easily done by analyzing multiple biopsies or subdivisions of a neoplasm. This could be done ex vivo for most neoplasms that are surgically removed, such as adenomatous polyps of the colon. It could also be done in vivo in head and neck neoplasms like oral leukoplakia [107,108], as well as other organs including bladder [68], lung [109,110], pancreas [111], colon [4], stomach [112], squamous esophagus [113], breast [2,114] and prostate [115].

The evolution of neoplastic clones is the basis for both progression to cancer and the emergence of therapeutic resistance. If we are to be successful in preventing or curing cancer, we will have to find methods to modulate that evolution. The first step in that effort is to understand clonal evolution in neoplastic progression. We are hopeful that we finally have the technology and scientific foundation to take those first steps.

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