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Tumor evolution: Linear, branching, neutral or punctuated?☆

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Abstract

Intratumor heterogeneity has been widely reported in human cancers, but our knowledge of how this genetic diversity emerges over time remains limited. A central challenge in studying tumor evolution is the difficulty in collecting longitudinal samples from cancer patients. Consequently, most studies have inferred tumor evolution from single time-point samples, providing very indirect information. These data have led to several competing models of tumor evolution: linear, branching, neutral and punctuated. Each model makes different assumptions regarding the timing of mutations and selection of clones, and therefore has different implications for the diagnosis and therapeutic treatment of cancer patients. Furthermore, emerging evidence suggests that models may change during tumor progression or operate concurrently for different classes of mutations. Finally, we discuss data that supports the theory that most human tumors evolve from a single cell in the normal tissue. This article is part of a Special Issue entitled: Evolutionary principles - heterogeneity in cancer?, edited by Dr. Robert A. Gatenby.

Keywords

Single cell genomics; Intratumor heterogeneity; Tumor evolution; Cancer genomics; Genome evolution; Cancer biology

1. Introduction

Tumor evolution begins when a single cell in the normal tissue transforms and expands to form a tumor mass. During this complex biological process, clonal lineages diverge and form distinct subpopulations, resulting in intratumor heterogeneity (ITH). ITH has long been observed by pathologists, such as Rudolf Virchow in the late 1800s who reported

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morphological differences between single tumor cells under the microscope [1]. Further development of karyotyping and cytogenetic technologies in the 1970s led to numerous studies reporting heterogeneity in amplifications of oncogenes and deletions of tumor suppressors within the same tumor [2–4]. The concept of ITH soon emerged, but was largely ignored in clinical practice, because it confounded the diagnosis and therapeutic treatment of cancer patients. In the late 1990s microarray technologies were developed [5], which were soon followed by the development of next-generation sequencing (NGS) technologies around 2005 [6,7]. These new genomic technologies led to a paradigm shift in the field, away from qualitative studies based on single markers, and towards large-scale quantitative ITH datasets. The subsequent application of NGS technologies to human tumors revealed that ITH is common in many human cancers [8–10]. However, despite the significant progress, a central question has remained: how did ITH emerge during tumor progression?

Tumor evolution is a field that applies knowledge of species evolution, ecology and population genetics to understand how tumor cell populations respond to selective pressures [11]. Formalizing the concept of tumor evolution is often accredited to Peter Nowell [12] and pioneers such as Isaiah Fidler who recognized the importance of clonal diversity in metastasis [13]. Over the following decades studies have showed that tumor cells encounter selective pressures in their microenvironment, including the immune system, pH changes, chemotherapy, radiation, nutrient deprivation and geographic barriers [14]. These selective pressures shape the evolutionary trajectory of the tumor and clonal lineages. Principles such as species richness, selection, fitness and population bottlenecks are useful concepts for understanding tumor evolution, however it is also important to note that many concepts from ecology and population genetics do not apply to tumors, most notably sexual selection and meiotic recombination [14,15].

Tumor evolution is difficult to study in human patients. The central problem is that patients cannot ethically be biopsied at multiple time points during the progression of the disease. As a consequence, most studies have inferred the evolutionary history from single time-point samples. This approach is conceptually feasible, because ITH provides a permanent record of the mutations that occurred during the natural history of the tumor [8,16]. Researchers can apply phylogenetic inference to reconstruct tumor cell lineages and order the chronology of mutations that occurred over time. However this approach provides an incomplete picture of how tumor cells evolve, particularly when intermediate clones are not persistent during progression. Consequently there has been much debate regarding the general models of tumor evolution. Several competing models have been proposed: Linear Evolution (LE), Branching Evolution (BE), Neutral Evolution (NE) and Punctuated Evolution (PE) (Fig. 1). The evidence supporting these models will serve as the basis of discussion for this review, but first we will review the genomic methods that are used to study ITH and clonal evolution.

2. Methods for resolving intratumor heterogeneity

NGS methods can measure thousands of mutations and generate large-scale genomic datasets on tumors [6,7]. However standard NGS methods require bulk tissue and therefore provide limited information on the subclonal architecture of a tumor. To address this

limitation, further methods were developed to delineate ITH: deep-sequencing, multi-region sequencing and single cell DNA sequencing (Fig. 2). Deep sequencing involves performing NGS at high coverage depth to measure mutant allele frequencies (MAFs) [17,18] (Fig. 2A). Using computational methods such as SciClone [19] or Pyclone [20], the mutation frequencies are then normalized and clustered to identify clonal subpopulations that are assumed to share similar MAFs. This approach is experimentally simple, but cannot accurately resolve clonal subpopulations when they share similar MAFs in the tumor. Another method is multi-region sequencing and involves sampling different geographical regions of the tumor for exome sequencing (Fig. 2B) [21–24]. This approach is experimentally straightforward, but has limited ability to resolve subclones that are intermixed within the same spatial regions. Another approach is single cell DNA sequencing (Fig. 2C) [25–29]. This approach involves isolating single tumor cells, performing whole genome amplification (WGA) and then sequencing and comparing multiple cells to resolve ITH and reconstruct clonal lineages [30]. The advantage of this approach is that it can fully resolve admixtures of clones, however due to cost and throughput, only a limited number of cells can be profiled, potentially leading to sampling bias [31].

3. Reconstructing tumor evolution from intratumor heterogeneity

After resolving ITH, the data can be used to reconstruct clonal lineages using phylogenetic inference to understand tumor evolution. In phylogenetic tumor trees, the internal nodes represent common ancestors, whose genotype can be deduced from the commonalities between their descendants. A phylogenetic tree thus provides a window into the past, by estimating the order in which mutations occurred as clones diverged in lineages and formed subpopulations. Phylogenetic trees can be constructed from ITH using different algorithms. The units of heterogeneity that appear at the tips of the tree are called *taxons*, and represent either clones, single cells, or spatial regions, depending on the experimental method that was used. The tree is often constructed using an algorithm to satisfy a *parsimony criterion*, in which the tree with the minimum number of changes leading to the observed data is inferred. For deep sequencing data, clones are inferred by clustering MAFs and are arranged into a tree using the *sum condition* that MAFs of child nodes must sum to those of their parents, and the *ancestry condition* that descendants have all the mutations in their parents. Many computational algorithms have been developed for this purpose [32–40]. There are also specialized algorithms for inferring phylogenetic trees from multiregion sequencing data [41,42]. Using single-cell data, it is possible to order mutations and attach cells to the mutation trees [43], or to additionally cluster cells into clones and construct a clone tree similar to those produced by deep sequencing analysis methods [44,45]. In summary, these methods enable tumor evolution to be reconstructed from ITH using single time-point samples. However these trees are based on the *infinite sites assumption* [46] which implies that mutations accumulate and are never lost. This assumptions is often violated in tumors, where chromosome deletions and LOH are common.

4. Evolutionary concepts and definitions

To understand models of tumor evolution, several concepts and definitions are necessary. A clone is defined as a group of tumor cells that shares a highly similar genotype and

mutational profile, while a subclone is a group of tumor cells that diverged in the evolutionary lineage and has acquired additional mutations [9]. Truncal mutations are ancestral mutations in the *trunk* of the phylogenetic tree that are shared by all clones, while *subclonal* mutations define a lineage that has diverged from the trunk [47]. *Private mutations* refer to mutations that are only detected in a single taxon. Another important concept is *fitness*, which refers to the ability of a tumor cell to survive and proliferate, so that it can propagate its genotype to the gene pool in the tumor. Tumor clones with increased fitness will become more prevalent in the tumor mass over time. *Driver mutations* confer a fitness advantage, while *passenger mutations* have no effect on fitness. Increased fitness can lead to clonal expansions in which one genotype expands in frequency in the tumor mass. A *selective sweep* describes the process in which a genotype emerges with an extremely high fitness that it outcompetes all other clones in the tumor [14].

5. Linear tumor evolution

One of the most renowned models for tumor evolution posited that mutations were acquired linearly in a step-wise process leading to more malignant stages of cancer [48]. In this linear evolution (LE) model new driver mutations provide such a strong selective advantage, that they outcompete all previous clones via selective sweeps that occur during tumor evolution (Fig. 1A). This model posits that selective sweeps occur after driver mutations are acquired, resulting in dominant clones when ITH is profiled at various stages of tumor growth (Fig. 3A). The resulting LE phylogenetic tree is expected to show a major dominant clone, with only rare intermediates that are persistent from the previous selective sweeps (Fig. 4A). Experimental evidence for LE was originally based on profiling X-inactivation in tumors using histological staining, methylation analysis or PCR genotyping of glucose-6-phosphate dehydrogenase [49–53]. These studies showed that unlike most somatic tissues, which had random inactivation of the maternal or paternal X allele, human tumors often showed only a single clonal X-allele inactivated throughout the tumor mass. These data were interpreted that human tumors were clonal growths, due to selection of the dominant clones. Further work by Fearon & Vogelstein showed that colon cancers progress through a linear series of step-wise mutations leading to sequentially more malignant stages of tumor growth in colorectal cancer [48]. Conceptually, this linear model has been useful in understanding how the sequential acquisition of driver mutations can potentially lead to more advanced stages of malignant disease. However most data supporting LE stems from single gene studies that did not measure genome-wide markers and thus may have missed heterogeneous mutations that define different clones. In summary, there is limited experimental evidence supporting LE in most advanced human cancers.

6. Branching evolution

Branching evolution (BE) is a model in which clones diverge from a common ancestor, and evolve in parallel in the tumor mass resulting in multiple clonal lineages (Fig. 1B). In contrast to LE selective sweeps are uncommon in BE, and multiple clones expand simultaneously because they all have increased fitness. In this model the amount of ITH will fluctuate during tumor progression, but multiple clones are expected to be present at the time of clinical sampling (Fig. 3C). The phylogenetic trees resulting from BE are expected to

have intermediate taxa as well as taxa that have clonally expanded due to the positive selection of driver mutations in subclonal lineages (Fig. 4B). The BE model has been supported by numerous NGS studies in which point mutations have been profiled by single cell DNA sequencing [25,28,29,54–57], multi-region sequencing [21,23,58–65] and deep-sequencing [17,18,66,67]. These studies have reported branching evolution in many human cancers, including leukemia [57,66] breast cancer [17,18,28,61], liver cancer [68], colorectal cancer [58,69,70], ovarian cancer [24,71], prostate cancer [64], kidney cancer [21,23], melanoma [72] and brain cancer [62,63,73]. Consistently, these studies have identified truncal mutations, subclonal mutations and private mutations in many human cancers.

While these studies often support BE, they differ in the number of clones that are reported and the shape of the evolutionary trees. In some tumors, the evolutionary trees have short trunks and many branches, indicating subclones with significant degrees of divergence. Long branches exist in some breast cancers and kidney cancers [21,28, 61]. Other trees have very long trunks and few branches, indicating subclones with many more similarities than differences, including breast and kidney cancers, as well as lung cancers [59,60]. The number of clonal subpopulations identified in BE also varies significantly in cancers and among patients with the same cancers. In a cohort of 104 triple-negative breast-cancer (TNBC) patients, resolving subclones with deep sequencing identified 1 to 19 subclones per patient [18]. Another study used multi-region sequencing of 50 breast cancers and identified only 1–4 major clonal subpopulations in each patient [61]. These data suggest a large amount of variation in the number of subclones that are present in human tumors, but this result may also depend on sequencing depth and the number of cells or regions that are sampled.

A defining feature of BE is ongoing clonal evolution, in which new driver mutations continue to accumulate gradually over time in tumor cells, leading to further clonal expansions within the tumor mass. Continued evolution and selection clones is supported by at least two lines of evidence: 1. subclonal driver mutations, and 2. convergent evolution. Subclonal driver mutations are frequently reported in multi-region and single cell sequencing studies of tumors. In one study, Wang et al. used single cell exome sequencing and identified subclonal driver mutations in *AURKA*, *TGFB2* and *CBX4* that lead to the clonal expansion of three subpopulations in patient with triple-negative breast cancer [28]. Another single cell exome sequencing study identified subclonal mutations in *APC* and *TP53* that lead to the expansion of a tumor subpopulation [56]. A larger multi-region sequencing study of breast cancer patients identified subclonal driver mutations in 13 out of 50 patients [61]. Similarly a large number of subclonal driver mutations have been reported in multi-region sequencing studies of kidney cancer [21,23], lung cancer [60], melanoma [72] and deep-sequencing of breast cancer genomes [17,18]. However other cancers such as lung cancer [59], glioblastoma [63], and liver cancer [68] have reported limited subclonal driver mutations. Another line of evidence for selection in tumor phylogenies is seen in cases of convergent evolution, where two independent lineages in the tumor mutate the same driver gene, leading to independent clonal expansions. Evidence for convergent evolution has been reported in 5 out of 10 cases of kidney cancer [60] and in 4 out of 50 cases of breast tumors [61], where multiple spatial regions had inactivation of the same cancer genes.

These data show that subclones can co-exist and expand in parallel and are not outcompeted by selective sweeps that dominate the tumor mass as posited by LE.

The co-existence of multiple subclones in BE raises interesting question about the possibility of clonal cooperation. Several recent studies have begun to investigate clonal interactions using *in vivo* systems to understand the functional significance of ITH. Marusyk et al. used an *in vivo* system and reported non-cell autonomous paracrine interactions, in which expression of IL6 increased the size of the tumor but not the size the secreting subpopulation [74]. In another study mammary mouse models with Wnt1 overexpression showed cooperative interactions between basal and luminal subpopulations that were codependent for driving tumor growth [75]. A different study used *in vivo* systems to show that small subpopulations of glioblastoma cells with mutated *EGFR* clones could promote the growth of neighboring wildtype cells through a paracrine interaction that involved LIF and IL6 cytokines [76]. These functional studies have begun to explain why multiple subclones co-exist in tumors, by revealing the cooperative interactions that drive tumor growth. However in other cases, tumor clones may co-exist due to a lack of direct competition, rather than cooperative interactions [77].

7. Neutral evolution

Neutral Evolution (NE) is an extreme case of branching evolution, which further hypothesizes that there is no selection or fitness changes during most of the lifetime of the tumor (Fig. 1C). This model assumes that random mutations accumulate over time leading to genetic drift and extensive ITH (Fig. 3C). Importantly, NE posits that ITH is a byproduct of tumor progression, and has no functional significance in driving tumor growth. The lineage tree resulting from NE will consist of many intermediate nodes and highly branched structure, without any evidence of single taxa that have expanded (Fig. 4C). NE was originally proposed in species evolution, challenging the idea of natural selection that is the cornerstone of Darwinian evolution [78,79]. As described above, natural selection is evidenced in tumors by subclonal driver mutations and convergent evolution. However, not all tumors have these features, leaving open the possibility of neutral evolution in some cases. Ling et al. used multi-region sequencing to profile 309 spatial regions of a single patient with a hepatocellular carcinoma and reported 35 polymorphic SNVs that defined 20 subclones. The frequencies of mutations among sections, and their spatial arrangements, were consistent with a mathematical model of neutral evolution in an expanding population [68]. In another study, Williams et al. applied a model of neutral evolution to examine subclonal allele frequencies from the TCGA cohorts and reported consistency with NE in one-third ($N = 323/904$) of examined cancers [80]. Evidence for NE in this cases was constituted by a linear relationship between the number of mutations and inverse mutant allele frequency. Together these results provide evidence that neutral evolution may occur in a significant fraction of tumors. However, this evidence has several limitations. Ling et al.'s report is a case study, and Williams et al. relies on low-depth exome data to detect subclonal mutations, which may have included many sequencing errors.

8. Punctuated tumor evolution

The linear, gradual and neutral models commonly assume that mutations are acquired sequentially and gradually over time. However, recent evidence has suggested that in some cancers, a large number of genomic aberrations may occur in short bursts of time, at the earliest stages of tumor progression (Fig. 1D). In this model ITH is very high at the earliest stages of tumor initiation, after which one or a few dominant clones stably expand to form the tumor mass (Fig. 3D). The resulting phylogenetic tree for PE is defined by the absence of intermediate taxa during tumor evolution, resulting a long root node and one or a few dominant clones (Fig. 4D). The term ‘Punctuated Evolution’ is borrowed from species evolution and was proposed by Gould and Eldredge as ‘Punctuated Equilibrium’ in 1970 to challenge the long-standing paradigm of gradual Darwinian evolution [81,82]. Importantly, punctuated equilibrium serves as an analogy to PE and the underlying mechanisms that cause rapid bursts of change are likely to be very different. Nevertheless, several key principles are relevant: 1. stasis (stable clonal expansions), 2. evolution in short bursts of time, and 3. lack of gradual intermediates that are persistent during evolution. PE is fundamentally different from the gradual tumor evolution models, since it posits that extensive ITH is generated at the earliest stages of tumor evolution, which is not ongoing during tumor progression. In this model, tumor cells are ‘hard-wired’ or ‘pre-programmed’ at the earliest stages of tumor growth and are therefore destined to become invasive, metastatic or resistant to therapy. This model has also been referred to as the ‘big bang’ model of tumor evolution [70]. In contrast to LE and BE, that are supported mainly by point mutations, PE has mainly been supported by experimental data on DNA copy number aberrations and chromosomal structural rearrangements.

The studies on punctuated evolution can be divided into two groups: 1) localized phenomenon on single chromosomes, or 2) whole-genome rearrangements leading to aneuploidy. One of the first observations of localized chromosome rearrangements was termed ‘firestorms’, and described patterns in which clusters of amplifications were constrained to single chromosome arms. These events were found to correlate with highly aggressive disease in breast cancer patients and were discovered by Hicks et al. using microarray CGH [83,84]. Subsequent work described a similar phenomenon called ‘chromothripsis’ in about 25% of bone cancers and 2–3% of other cancers using paired-end sequencing [85,86]. Chromothripsis is more specifically defined by many oscillating copy number states in which breakpoints map between adjacent segments on a single chromosome, and has been reported in colorectal cancer [87], prostate cancer [88], and ovarian cancer [89], among other cancer types [90,91]. Although many studies speculated that chromothripsis is caused by erroneous non-homologous end joining (NHEJ) after DNA damage from sources such as ionizing radiation, a recent single cell DNA sequencing study by Zhang et al. showed that it may also be caused by micronuclei formation [86].

PE has also been implicated in the genesis of genome-wide aneuploidy. The genomic data supporting a model of punctuated copy number evolution (PCNE) comes from multiple studies using NGS, single cell DNA sequencing and microarray CGH [25,29,83–85,92,93]. One of the first studies to report PCNE used a method called Sector-Ploidy-Profiling (SPP) that combined tumor macro-dissection, flow-sorting by DNA ploidy and microarray CGH to

infer copy number lineages from 20 patients with invasive breast cancer [92]. In another study, the authors developed the first single cell DNA sequencing method and profiled genome-wide copy number in hundreds of single cells from two invasive breast cancer patients, revealing phylogenetic lineages consistent with PCNE [25]. A more recent study used high-throughput single cell DNA sequencing methods to profile thousands of single cells from 12 patients with TNBC and reported that PCNE was common in this subtype of breast cancer [29]. In prostate cancer a study of 57 patients, another group reported a phenomenon called ‘chromoplexy’, in which genome-wide translocations and CNAs were interdependent and occurred concurrently in short bursts of time [93]. In colon cancer, a study by Sottoriva et al. profiled CNAs, epigenetic markers and mutations in the spatial distribution of 349 individual glands (b1000 cells) of 15 colorectal cancer patients and reported a ‘big-bang’ model in which all of the mutations were hypothesized to occur at the earliest stages of tumor initiation [70]. Taken together, these studies suggest that CNAs and chromosome structural rearrangements may evolve through a PE model of tumor progression.

9. Computational modeling of tumor evolution

A limitation in the field of tumor evolution has been that most studies are based on a single time point samples, making it difficult to reconstruct clonal dynamics over time. To address this problem, mathematical models have been developed that simulate cancer evolution [28,29,68,80,94–110]. These methods allow adjustments of parameters to better understand how they affect ITH and clonal evolution. Multiple studies have analyzed ITH using multitype branching process models [29,94–97]. These models make two primary assumptions: 1) that the probabilities that a cell divides or dies are independent of all other cells, and 2) that each cell division may result in a mutation, which produces a cell of a different ‘type’, with different division and death rates. Another simple model, the Moran model, adds the assumption that each cell division is accompanied by a cell death [98]. Whereas branching processes provide good models of exponentially growing populations, the Moran model is a better model for a population of fixed size. Many variations on these models exist, with different assumptions about details such as the time between cell divisions and the number and nature of types. For example, the near critical age-dependent branching process provides a model of a tumor as it approaches a steady state population size [99]. Instead of a succession of types, complex forms of mutation such as gain and loss of double-minute chromosomes can be accommodated [100]. More complex models can be used to incorporating elements such as spatial limitation and microenvironment heterogeneity. Very complex models are difficult to mathematically analyze and are usually studied with computer simulations [101].

Mathematical modeling has been integral to the discovery of punctuated and neutral evolution. To determine the model of evolution governing a hepatocellular carcinoma, mutation frequencies in multiregion sequencing were compared to predictions from a neutral model [94], revealing a quantitative consistency that evidenced a history of neutral evolution [68]. Spatial arrangement of genotypes was also consistent with a spatial model of neutral evolution [68]. In a pan-cancer analysis of neutral evolution, MAFs from TCGA data were compared to quantitative predictions from a branching process model, with consistency with

the neutral model found in about a third of examined cases [80]. To determine if evolution in TNBC is punctuated or gradual, simulated single-cell sequencing data was produced from four variants of the multitype branching process model: one in which mutations are random and independent, one modeling epistatic interactions between mutational fitness, one in which there was a temporary global increase of the mutation rates, and one in which many mutations could be acquired in a single burst [29]. Only the last model with bursts of mutations was able to recapitulate the punctuated tree structures observed in single-cell copy number profiles of 12 TNBC tumors, whereas other gradual models were found unable to achieve the long-stemmed clonal trees in a reasonable physiological timeframe. Chromoplexy in punctuated evolution was evidenced by the inability of a model of gradual accumulation of structural variation to explain observed co-occurrence of breakpoints [93]. Future work may take advantage of advances in phylogenetic inference to test models using statistics defined by the phylogeny, such as average time to common ancestor, which is informative of the growth rate of the cells [99].

In addition to analyzing single time-point data, mathematical models also provide a theoretical understanding of the conditions that favor branching evolution. In a Moran model in which mutations produce new types with random fitness changes, when population size and mutation rate are sufficiently small, mutation frequencies go to 0 or 1 before the next mutation occurs, resulting in linear evolution [102]. Iwasa and Foo studied heterogeneity both mathematically and by simulation in a Moran model with both passenger and driver mutations. They performed simulations using higher mutation rates than those expected to lead to linear evolution, resulting in persistent ITH despite selection [103]. Other modeling studies have examined the effect of spatial limitations, which tend to increase ITH by limiting the rate of expansion of new clones [68,104–107]. More complex models of tumor evolution have also included cell migration [106–108], which mitigates the ITH increase caused by spatial limitations [107], and heterogeneity in the microenvironment [109].

A surprising result from mathematical modeling is that, even if fitter populations completely displace less fit ones, linear evolution does not necessarily follow. The reason is that mutations providing similar fitness increases may emerge at similar times in different cells of the tumor, so that the new population does not originate from a single driver event but a patchwork of driver events that provide similar fitness advantages. This phenomenon has been studied in multitype branching processes, by considering independent mutations to the same type to result in different ‘labels’. Durrett et al. calculated the expected value of a diversity index for labels within a type, and found that expected diversity was high when the fitness increases provided by a mutation are low [94]. Since fitness increases after tumor initiation may be low [110,111], this provides a theoretical explanation of branching evolution, and is consistent with observations of different driver mutations in different subclones. In a different multitype branching process, McDonald and Kimmel have provided a detailed mathematical characterization of the frequency distributions of labels [96].

A major limitation of mathematical models is that the results depend on the values of difficult-to-determine parameters. These parameters include cell division rate, cell death rate, tumor size, population size, mutation rate, and the fitness advantage conferred by driver mutations. Parameters such as cell birth can be measured with immunohistochemistry using

Ki-67 staining, while parameters for cell death can be measured with Caspase-3 or TUNEL assays [28,29,108]. Mutation rates can be estimated in cell lines through single-cell sequencing if the number of generations is known precisely [27]. Incorporating experimentally derived parameters can greatly improve models that simulate tumor growth and ITH. However more indirect, model-dependent estimation is required to estimate mutation rates [28,80], fitness changes [111], and early birth and death rates [112] in human cancers. Selective sweeps can be detected by changes in effective population size, which can be estimated mathematically from variant frequency distributions [113]. Another important consideration is the inter-patient variation in parameters, which can show broad ranges in within a very specific cancer type. In the modeling of disease progression in patients with acute myelogenous leukemia it was necessary to model distributions of proliferation rates that vary in patient populations [114]. Similarly, modeling variation in cell proliferation and death rate parameters in melanoma patients in response to therapy was required to fit the distribution of changes in tumor size [115].

10. Mixed models of tumor evolution

Although most studies have reported a single model of tumor evolution, there is emerging evidence that models may undergo transitions over time, or that multiple models may be operating concurrently for different classes of mutations. While LE has not been supported by studies in advanced carcinomas, mathematical modeling suggests that this model may occur at the earliest stages of tumor progression in small, fixed population sizes [102]. Thus tumor evolution may shift from LE to BE as the population size continues to expand. More work will be needed to compare early stage cancers (e.g. colon adenomas, ductal-carcinoma-in-situ, prostatic intraepithelial neoplasia) to advanced carcinomas to determine if LE is more common in early neoplasias. Similarly, it has been proposed that tumor evolution may shift from PE to NE in colon cancer [116]. This hybrid model suggests that all driver mutations are acquired in the initial stages of tumor evolution, after which clones expand without further selection. An interesting observation in the literature is that much experimental data suggests that point mutations follow a BE model, while CNAs and chromosomal structural variants follow a PE model. These models may not be mutually exclusive, but instead operate simultaneously during tumor progression as two independent molecular clocks. Studies using single cell copy number pro-filing and single cell exome sequencing from the same breast cancer patients, have shown that CNAs occur in early punctuated bursts of evolution, and stably expand, while point mutations evolve gradually over the lifetime of the tumor leading to clonal expansions [28]. Other studies using whole-genome sequencing of matched longitudinal breast tumor samples of hyperplasias, DCIS, and invasive carcinomas, have also shown that copy number evolution occurred at the earliest stages of progression, while point mutational evolution was more gradual and branching throughout the course of the disease [117]. Collectively these studies make sense in the context of the molecular mechanisms that underlie the mutational processes. Complex aneuploid copy number changes may occur in just a few cell divisions due to mechanisms including endoreduplication, telomere attrition, breakage-fusion-bridge and chromosome mis-segregation events [118–120]. In contrast mutations accumulate gradually over time due

to defects in DNA repair pathways and errors in DNA polymerases, since most mutations are passenger events with no effect on fitness [121,122].

11. Tumors evolve from single cells

A long standing debate in the cancer field has been whether human tumors originate from a single normal cell, or alternatively multiple initiating cells that give rise to the same tumor mass [123]. In cancers that are multifocal (e.g. prostate, liver cancer), or where there are known exogenous mutagens (UV, cigarette smoke) or germline mutations (*BRCA, TP53, APC*) many studies have proposed the concept of a cancer 'field effect' that can give rise to multiple initiating cells [124]. However, a surprising result of all the tumor evolution studies published to date from deep-sequencing, multi-region sequencing and single cell sequencing has been that almost every patient's tumors have a shared set of truncal mutations that indicate a common evolutionary origin: a single normal cell. Numerous truncal mutations have been reported in primary tumors from breast cancer [17,18,61], prostate cancer [64], lung cancer [59,60], melanoma [72], colon cancer [69,70], pancreatic cancer [65], brain cancer [62,63,73] and bladder cancer [55]. What is perhaps unexpected is that even solid tumors such as lung cancers, where cigarette smoke is expected to cause a field effect in the lung epithelium, multi-region sequencing data has shown that all clones share a common evolutionary lineage and origin [59,60]. Similarly, in melanoma, where UV causes a field effect across the skin epithelium, multi-region sequencing has shown truncal mutation in 41 biopsies from 8 patients, indicating evolution from a common origin [72].

These data suggest that the initiation of a tumor cell that can progress to a carcinoma is an extremely rare event, that only occurs once in the lifetime of most cancer patients (if it all). Studies using ultra-deep sequencing at higher sensitivity have reported rare subclonal expansions in potentially thousands of pre-malignant clones in eyelid cancers that were exposed to a lifetime of UV radiation, however these pre-malignant clones never progressed to an invasive tumors [125]. While the majority of cancer patients have shown evidence of a single cell ancestor, there has been a single report of a patient with independent tumor lineages contributing to the same tumor mass. In multifocal prostate cancer 1/5 patients was found to have no truncal mutations between two different geographically sequenced regions of the tumor mass, suggesting at least two independent initiating cells [125]. Collectively these data suggest that the vast majority of solid tumors initiated from a single normal cell that diverged and branched to form the tumor mass.

12. Clinical implications

Models of tumor evolution have different implications for the clinical diagnosis, prognosis and therapeutic treatment of cancer patients. From a diagnostic standpoint, LE and PE imply limited ITH at the time of clinical sampling, which simplifies diagnostic assays because single biopsy samples are representative of the tumor as a whole. In contrast both the BE and NE suggest that ITH is extensive and would require multi-sampling approaches from different spatial regions to detect all of the clinically actionable mutations in the tumor. Alternatively, single tumor samples using large pieces of tissue can be used for targeted deep-sequencing (N1000X) to detect subclonal mutations, as implemented by cancer gene

panels such as Foundation One [126] or the MD Anderson Institute for Personalized Cancer Therapy T200 Panel [127].

Intratumor heterogeneity may prove useful as a prognostic or predictive biomarker [128]. A 'diversity index' from a patient's tumor may be useful for predicting poor survival, poor response to therapy, or higher risk of metastasis or relapse. Data from Almendra et al. showed that breast cancer patients with lower copy number ITH were more likely to have complete pathological response to neoadjuvant chemotherapy [108]. Similarly, in head and neck cancers, increased ITH was shown to correlate with poor survival [129]. Another study showed that increased ITH is associated with higher probability of progression from Barrett's esophagus to esophageal cancer [130]. Other studies have shown that multiclonal tumors with increased ITH are more likely to evolve resistance to therapy [129,131–133]. Notably, a strict NE model would imply that ITH has no clinical significance, since this model postulates that diversity is a by-product of tumor evolution that does not confer a fitness advantage to tumor cells [116].

Models of tumor evolution also have relevance to the therapeutic treatment of cancer patients. In LE and PE, the clonal subpopulations are assumed to be homogeneous, which would suggest that most CNAs or mutations can be targeted to eliminate the tumor mass. However, the BE and NE models imply extensive ITH that varies spatially throughout the tumor mass. In these models it would be necessary to target truncal driver mutations that occurred early in tumor progression and were subsequently inherited by all cancer cells [9,47,77,134]. This approach is currently being implemented in a large multi-region sequencing clinical trial of lung cancer patients called TRACERx [135]. Another therapeutic strategy is to target subclonal mutations to eliminate minor clones that may play an important role in invasion, metastasis or therapy resistance [136]. In a recent study Yates et al. used multi-region sequencing and found that 13/50 patients had clinically actionable subclonal mutations [61]. Targeting subclones may potentially lead to the extinction of the entire tumor mass if clones cooperate and are interdependent for driving tumor growth [74,75]. In contrast, NE assumes that targeting subclonal mutations is futile, since they do not provide a fitness advantage to tumor cells and are thus unlikely to be driving the cancer.

An alternative therapeutic approach is anti-evolution therapy. The conceptual basis of this approach involves targeting the mechanisms that fuel evolution, rather than the end-product (ITH). Lessons from species extinction in paleontology may provide insight into treating tumors, since extinction is rarely caused by a single selective pressure, and geographic dispersion plays an important role in preventing extinction [137]. Thus targeting multiple selective pressures in combination may be needed to eradicate a tumor mass. Another evolutionary approach involves directing evolution towards a specific trajectory that will sensitize the tumor cells to subsequent drug treatments. This strategy was demonstrated using two therapies, where the first drug (anti-p53 vaccine) was introduced to increase the sensitive of residual cell populations to the second therapy (chemotherapy) [138,139]. Stress in the tumor microenvironment, such as hypoxia, pH changes and limited nutrients can accelerate evolution by leading to increased mutation rates or genome instability [140]. To mitigate stress responses in tumor cells, targeting hypoxia [141] or metabolism [142,143] have been proposed. Another approach proposed by Gatenby and colleagues [144] called

adaptive therapy, involves administering low-dose chemotherapy over extended periods of time under the framework of metronomic therapy to keep the tumor volume at a constant level, treating it as a chronic disease [145–147]. The evolutionary reasoning behind this strategy is based on a food chain, in which the goal is to keep the population sizes of dominant tumor clones (apex predators) in check so that minor species (resistant clones) do not expand in their place [148–150]. Other ecological strategies have involved targeting the tumor stroma and tumors cells concurrently to inhibit microenvironment interactions that are required for continued tumor growth [151,152].

13. Conclusions & future directions

In conclusion, the literature published to date supports a BE model for point mutations, and a PE model for copy number evolution. In contrast there is limited data supporting a neutral or linear evolution model, but notably there are a limited number of studies on NE. However, an important bias of most tumor evolution studies is that they have focused on the analysis of advanced, high-grade tumors, since large amounts of tissue were necessary for NGS methods. Therefore, tumor evolution models in early stage cancers remain understudied. While two papers have reported some evidence for NE [68,80], these studies are in conflict with a large body of literature that shows subclonal expansions in tumor lineages after specific driver mutations have been acquired during BE [21,23,28,55,60]. Future work will be needed to determine if selection and fitness changes are ongoing during tumor evolution, to better distinguish between these models.

An important conclusion from this review is that individual tumors may not follow a single model of tumor evolution. Instead several studies have suggested that multiple models may be operating at different stages of progression, or concurrently during tumor evolution. Mathematical and computational modeling shows that LE may occur at the earliest stages of tumor evolution, after which BE may take over when the tumor is actively growing [94,102]. Other studies have suggested mutations may occur through a PE model that is subsequently followed by NE in colon cancer progression [153]. Studies have also begun to show that PE and BE may occur concurrently during tumor evolution, representing two distinct molecular clocks for copy number evolution and point mutation evolution [28,93]. Future work will be needed to determine if point mutations, copy number changes structural variants, indels and epigenetic events follow different models of tumor evolution and operate concurrently in the tumor mass.

The central problem with most tumor evolution studies is that they were based on the genomic analysis of single time-point samples. Future work should be directed towards obtaining multiple longitudinal tumor samples to study clonal dynamics over time and in response to therapy. This work can be performed either in human patient samples, or using *in vivo* experimental systems, such as xenografts [154]. The major challenge with collecting longitudinal samples from human patients is that biopsies require invasive procedures. An exciting technological advance is the development of ‘liquid biopsy’ methods to isolate and profile circulating tumor cells (CTCs) [155–157] and circulating tumor-DNA (ctDNA) [158,159]. These methods can be performed on patients during multiple time-points during the progression of their disease using non-invasive blood samples. However, to date most

studies of CTCs and ctDNA have focused on tracking single targeted mutations or limited gene panels [159–161]. These approaches are not ideal for resolving ITH and clonal substructure, where thousands of markers are needed. However, recent studies have demonstrated the technical feasibility of performing whole-exome profiling of single CTCs [155,156] or ctDNA [162,163]. These methods will provide a unique opportunity to monitor genome evolution in 'real-time' to improve our understanding of tumor evolution.

Another approach for collecting longitudinal samples is *in vivo* systems, such as patient derived xenografts (PDXs) [154]. PDX mice are ideal systems for studying ITH, since they conserve the clonal diversity that was present in the original human tumor. NGS studies have shown that the genomic profile of matched primary tumors and xenograft passaged tumors have a high concordance of somatic mutations, suggesting that xenografts represent physiologically relevant systems for studying tumor evolution [164–166]. A recent study demonstrated the utility of using xenograft systems to study tumor evolution in TNBC using deep-sequencing and single cell sequencing methods to monitor clonal dynamics during multiple passages of PDX tumors [167]. However xenografts also have several technical limitations, including the lack of a functional immune system and a mouse stroma. These issues are beginning to be addressed by implanting tumors into physiologically relevant organ sites and reconstituting the immune system in immunocompromised mice after transplantation [168].

In closing, the current literature suggests that advanced carcinomas follow a branching evolution model for point mutations, and a punctuated evolution model for CNAs. While conceptually it is useful to distinguish these models, it is important to note that in reality multiple models may be operating concurrently. Future work using longitudinal samples from human patients, liquid biopsies and PDXs will provide more insight into these models and their relevance to human cancers. This work is justified from a clinical standpoint, since it has important implications for the diagnosis and therapeutic treatment of cancer patients. In the near future, we predict that clinical assays will soon be designed to account for ITH through the translation of novel technologies, such as single cell sequencing and multi-region sequencing. Furthermore, we anticipate that new therapeutic strategies will shift from targeting the end-product of tumor evolution (ITH), towards the evolutionary mechanisms that give rise to clonal diversity in the first place. These efforts will undoubtedly lead to more effective treatments and prevent the ability of tumor cell populations to evolve resistance to therapies.

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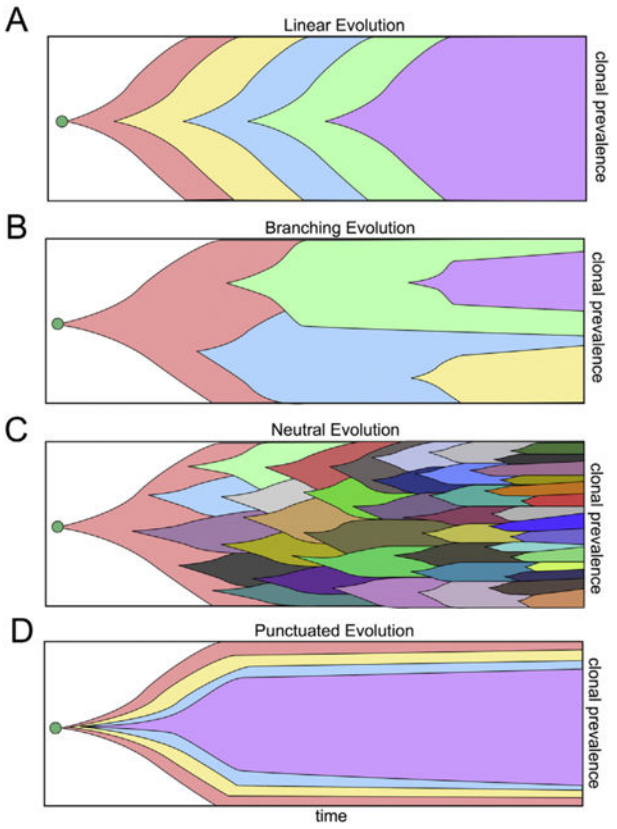

Fig. 1.

Models of tumor evolution. Illustration of tumor evolution models showing dynamic changes in clonal frequencies over time. This figure is based on the original publication by Marusyk and Polyak [8]. (A) Linear Evolution (B) Branching Evolution (C) Neutral Evolution (D) Punctuated Evolution. Colors indicate clones with different genotypes.

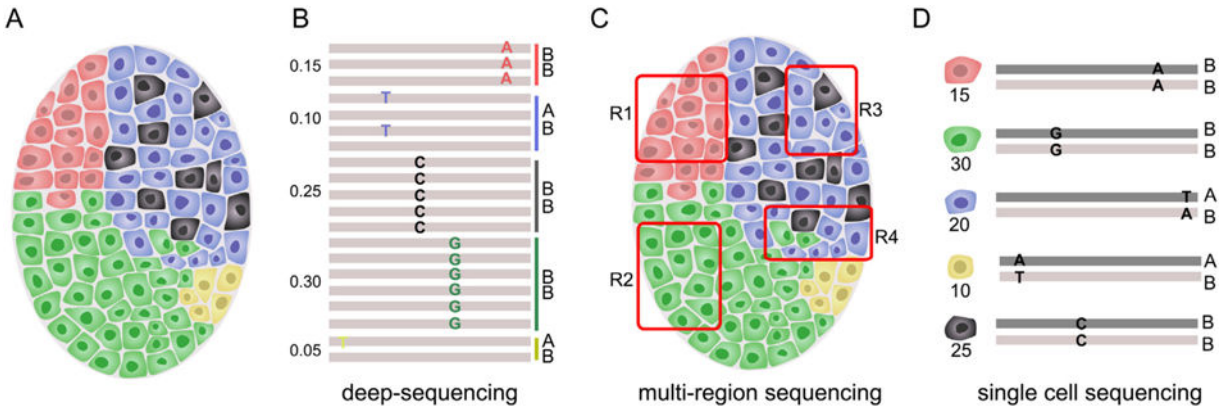

Fig. 2.

Sequencing methods for resolving intratumor heterogeneity. NGS methods for resolving intratumor heterogeneity. (A) Heterogeneous tumor with five clonal subpopulations indicated by different colors (B) Deep-sequencing and clustering of mutation frequencies (C) multi-region sequencing of different spatial regions in the tumor mass (D) single cell DNA sequencing of individual tumor cells isolated from the tumor.

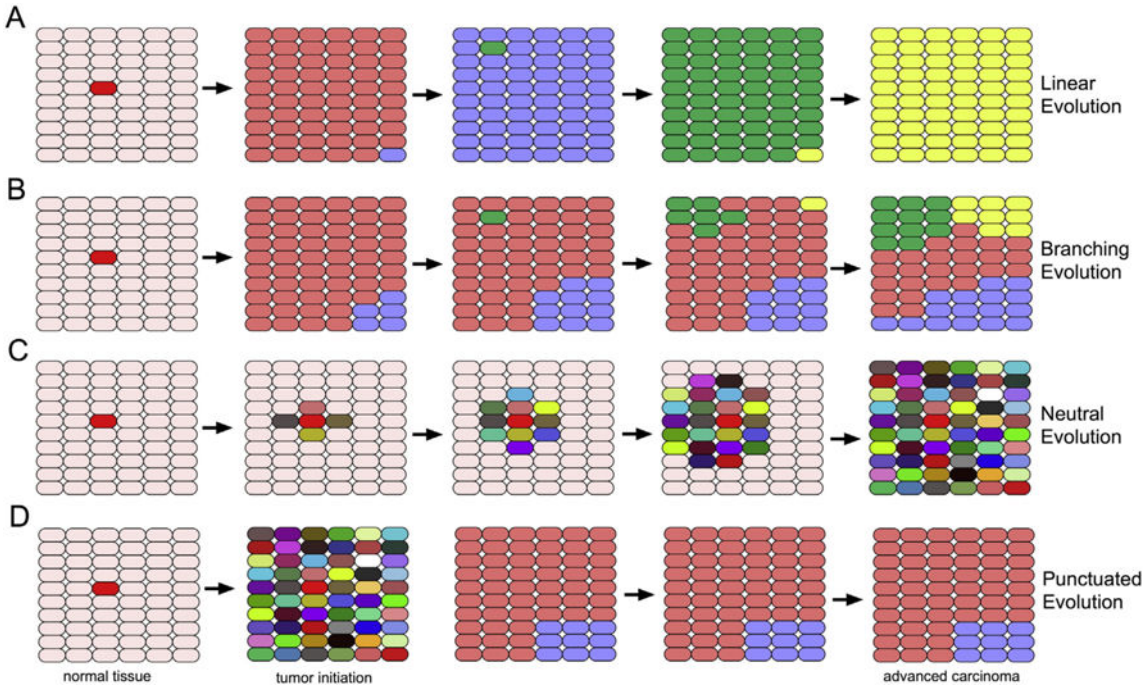

Fig. 3.

Progression of ITH in tumor evolution models. Changes in intratumor heterogeneity during tumor progression in the context of different tumor evolution models. (A) Linear evolution (B) Branching Evolution (C) Punctuated Evolution (D) Neutral Evolution. Colors indicate different genotypes of clones.

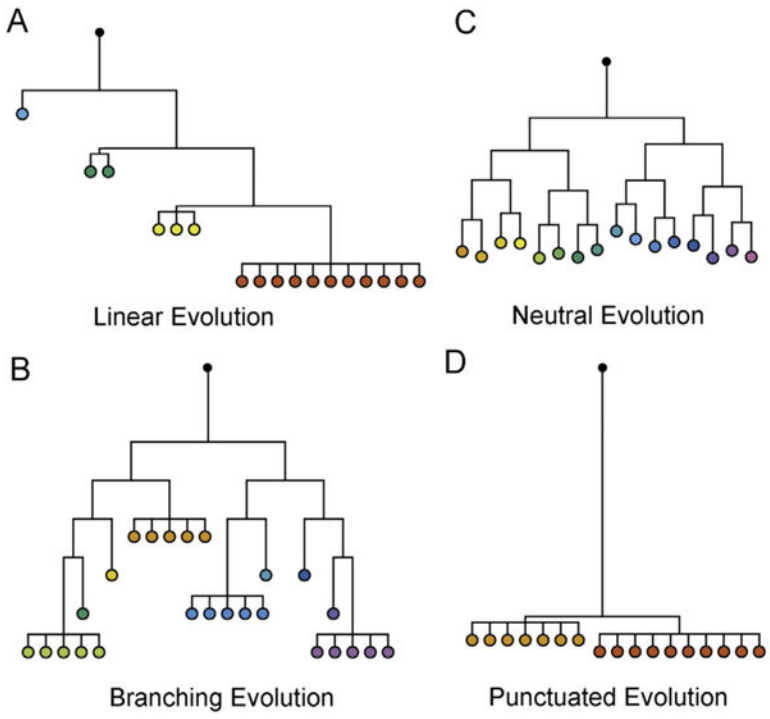

Fig. 4.

Clonal lineages and phylogenetic trees. Phylogenetic trees expected from different models of tumor evolution (A) Linear Evolution (B) Branching Evolution (C) Neutral Evolution (D) Punctuated Evolution. Colors indicate clones with different genotypes.