Resolving genetic heterogeneity in cancer

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Abstract

To a large extent cancer conforms to evolutionary rules defined by the rates at which clones mutate, adapt and grow. Compared to species evolution however, cancer is a particular case, due to the vast population size, chromosomal instability, and the potential for phenotypic plasticity. Nevertheless, an evolutionary framework is a powerful aid in our understanding of cancer progression and therapy failure, and could be applied to predict individual tumour behaviour and aid treatment strategies.

Introduction

Tumours are composed of subpopulations of cells (subclones) that may be distinguished by a variety of features impacting their phenotype, including genetic alterations. Genetic intratumour heterogeneity (ITH) has been documented across most cancers (reviewed in[1]) and acts as a substrate for clonal evolution. The fundamental biological mechanisms underlying clonal evolution **[G]** in cancer are similar to those that underpin the evolution of asexually-reproducing species: replication, heritable variation, genetic drift **[G]**, selection **[G]** and environmental changes. Central to the neo-Darwinian synthesis of evolutionary biology is the paradigm of molecular evolution **[G]**, which links Mendelian genetics to Darwinian adaptation. Molecular evolution is relevant to cancer because the use of genomic sequencing is a key technology to understand temporal and spatial patterns of somatic evolution **[G]**. At the core of molecular evolution, in turn, is theoretical population genetics, which has been the fundamental mathematical formalism to describe evolution for the past 90 years [2,3] The same theoretical framework has been used to understand clonal evolution in cancer [4] [5] [6] [7] [8] [9] [10] [11]. The study of the evolutionary dynamics of cancer clones is fundamentally concerned with the relative frequencies of cancer subpopulations over space and time. Although some peculiarities of cancer evolution distinguish it from classic species evolution (Box 1), classical evolutionary theory can nevertheless be readily applied to understand cancer development.

Over the past 5 years, a number of next generation sequencing studies have captured cancer evolution in space and time, illuminating the variety of evolutionary patterns that shape cancer, and showing their clinical relevance. Here, we provide an overview of the theoretical models of tumour evolution and the caveats around correctly interpreting genomic data and inferring evolutionary dynamics. We discuss the relevance of chromosome instability **[G]** (CIN) as a driver of cancer evolution and, in particular, metastases; the clinical value of evolutionary classification of cancer; and finally, the role of clonal evolution in treatment failure.

Current models of tumour evolution

Cancer as a system is characterized by an astonishing complexity and emergent behaviour. Nevertheless, this complexity arises from the relatively simple, underlying evolutionary rules of mutation, genetic drift and selection, involving a large number of interacting agents (for example, the millions of cancer cells within a single lesion and the surrounding tumour microenvironment). The emergent behaviour gives rise to different observed 'modes' of evolution (**Figure 1**), which result from different combinations of the aforementioned fundamental rules in distinct contexts. In other words, since selective pressures change over time, so can the 'modes' of evolution. Here we discuss the principles of selection and different modes of evolution.

Selection

Selection, whereby one lineage is 'favoured' over another, and produces more surviving offspring, is arguably the most interesting force in evolution, as it leads to adaptation. In general, positive selection drives tumour progression, but negative selection (e.g. against potent neoantigens [12,13]) also contributes to tumour evolution. However, selection is not be operative at all times. Whereas mutation and drift are continuously occurring, and their rate depends on cell division and population dynamics, selection is dependent on the environmental context. For example, if there is no differential survival within a population, the lack of positive selection would mean that the population evolves neutrally (only mutation and drift are at play). Consequently, branching (see below) of a tumour phylogenetic tree **[G]** does not always imply clonal selection, as branching is the natural product of mutational processes [14 15]. Selection has the effect of 'pruning' the tumour tree, for example, favouring the expansion of some lineages (branches) over others. The mutation rate itself could also be subject to selection. A higher mutation rate allows for diversification but also carries the 'risk' of increasing the rate at which deleterious mutations, which perturb cancer growth, are acquired[16,17]. For example, excessive chromosomal instability (CIN) can result in cell autonomous lethality, however a “just-right” threshold of CIN may be evolutionary advantageous. Mutations in the APC/C subunits may be selected during the evolution of chromosomally unstable tumour cell populations resulting in lengthening of mitosis, suppression of chromosome missegregation and attenuation of excessive CIN[18].

Mathematical models suggests that, in a growing population, mutator phenotypes are selected, because the cells that stochastically acquire positively selected mutations in effect ‘doubly benefit’ from their own increased fitness and the negative fitness effect of deleterious mutations on the rest of the population[19]. Relatedly, modelling also suggests that a mutator phenotype increases the ‘efficiency’ of carcinogenesis by making it more likely that a necessary set of mutations are acquired for transformation and cancer progression[17].

Branching evolution

Evolution is always branched, because cell division and mutation continuously produce divergence at the level of genotypes. This fact is particularly true for cancer genomes, as cancers often have a mutator phenotype [G] [20]. Hence, in principle, at any given time point a tumour cell population consists of different cell lineages. Random fluctuations in the birth and death rates of these distinct lineages can lead to genetic drift, whereby one lineage produces more surviving offspring than another lineage, and expands by chance. Genetic drift is referred to as a form of neutral evolution [G], as all lineages are neutral other with respect to their chance of producing surviving offspring [21,22]. Similar patterns of branching are also apparent in healthy tissue [23,24], emphasising branching as a necessary by-product of proliferation tissues. However, when multiple cancer subclones have increased fitness they will expand (assuming no other limitations to growth) simultaneously due to selection, as evidenced by the finding of subclonal cancer driver mutations and their impact upon cancer progression[25,26]. Selection is also evident in the finding of parallel evolution within the same tumour where distinct lineages acquire mutations in the same cancer driver gene, leading to parallel subclonal expansions.

Linear evolution

Linear evolution model posits that only one of the lineages survive over time. However, as with the fossil record, it does not imply that there was only ever a single lineage that evolved in a step-wise fashion. Any conclusions regarding linear evolution from cancer genomic data are likely false owing to limited sampling applied to the cancer in question and limits of resolution by next-generation sequencing technologies.

Neutral evolution

Neutral evolution can be regarded as the evolution that occurs in between selection events. Prior to adaptive mutation occurring, the population evolves neutrally, and when the mutation arises it initiates a rapid clonal sweep **[G]** which can be complete or incomplete. If the sweep is complete and all the cells in the population carry the adaptive mutation the dynamics revert to neutral again.

Punctuated evolution

Punctuated evolution is the opposite of gradual evolution and presents more rapid bursts of adaptive evolution. If the adaptive mutation is a large-scale alteration of the genome (for example, loss or gain, translocation or fusion of a chromosome) the adaptive clone has been referred to as a "hopeful monster" [27,28] . Compared to a small-scale mutation, its genome is significantly altered, with the 'hopeful' referring to the likelihood that the mutation is adaptive. Punctuated equilibrium **[G]** is a model first proposed by Eldridge and Gould in the early 1970s for species evolution [29] whereby adaptation occurs in a small spatially-isolated niche, until the newly-adapted individuals rapidly expand out of the niche and through the wider population. Because the niche is small, the gradually-adapting population is unlikely to be sampled before it expands, and so the evolutionary dynamics of the population at large are 'punctuated' by the expansion of the adapted clone. Equilibrium refers to long periods of clonal stasis during which the adapted clone persists without detectable change.

Inferring evolutionary mode from genomic data

Although adaptation occurs at the phenotypic level, measuring the tumour cell phenotype within its original environment is challenging. Surrogate measurements such as gene expression are informative, but given the complexity and plasticity of the cancer transcriptome, and the contribution to gene expression signals from cells within the tumour microenvironment, these are often difficult to interpret in light of evolution. This is why to date genome profiling has been the preferred tool to study cancer evolution. However, there are several major caveats when we try to understand the phenotypes from studying the genotypes, a problem that has been tackled over decades in the field of molecular evolution. The key issue is that the cancer genotype–phenotype map **[G]**, bar some notable exceptions, such as treatment resistance mutations, is largely unknown. Therefore, mapping the tumour phylogenetic tree and the underlying adaptive traits remains difficult.

Bulk sequencing

The commonly used bulk sequencing, that is profiling of a sample comprised of many cells, imposes a major limitation on inferences about tumour evolution dynamics. Because the standard depth of sequencing is many orders of magnitude smaller (100–1000X) than the number of cells in the sample (10 million – 1 billion), bulk sequencing only recovers mutations that are either present in all, or the majority of cancer cells in the given sample. Each doubling of the cancer cell population halves the frequency of new mutations arising in the population, and hence after just 7 doublings new mutations are undetectable with 100X sequencing, and after 10 doublings new mutations are undetectable at 1000X sequencing depth. Thus, detecting selection that resulted in a limited clonal expansion (100s-1000s cells) is problematic. Contamination by stromal cells imposes an additional challenge as it dilutes out the frequency of variant alleles. Thus, bulk sequencing mostly informs on the most recent common ancestor (MRCA) of the cells in the sample, but the 'node' in the phylogenetic tree is extinct in the current malignancy. The more cells in the bulk sample, the older the MRCA and shorter the 'apparent' branches in the tree. Consequently, different-sized samples can generate very different portraits of the clonal structure of a tumour.

Choice of sequencing assay

The relative abundance of passenger mutations **[G]** (evolutionary neutral; non-adaptive) over driver mutations **[G]** (positively selected) makes the passengers that hitchhike to a driver event, very informative vis a vis clonal dynamics. Passenger mutations provide a genetic mark to distinguish different functional clones, and more specifically the number of passenger mutations unique to a lineage is a measure of the molecular age of that clone. The variant allele frequency **[G]** (VAF) determines clone abundance, and the proportion of passenger mutations shared between clones reveals their ancestry [8,30]. The choice of sequencing assay (high-depth targeted panel, moderate-depth exome, or lower-depth whole-genome sequencing) represents a trade-off between the need for high-depth sequencing to accurate recover clone frequency (or even detect the clone at all) versus genome-wide detection of passenger mutations that uniquely identify distinct clones. Moreover, since deeper sequencing provides a broader temporal window on cancer evolution, the choice of sequencing assays is a compromise between genome sequencing providing detail on the clonal architecture in only a short and early time window versus deep targeted sequencing that provides limited clonal information but greater temporal range. Here, deeper and broader (for example, more of the genome covered) sequencing is always preferred.

Allelic copy-number correction

The study of evolutionary dynamics of cancer clones is fundamentally concerned with the relative frequencies of cancer clones over space and time. Many bioinformatics tools have been created to infer clonal frequencies from 'bulk' sequencing data, such as PyClone[31], SciClone[32] and PhyloWGS[33]. Broadly, these tools attempt to identify sets of mutations that are all at the same frequency, and assign them to clones. These tools have been instrumental to study cancer evolution from cancer bulk data. However, this task requires many prior inference steps, each one risking introduction of errors, which are then propagated through the analysis. Structural alterations (loss, gain and rearrangements of genetic material) are common in cancer genomes and confound the interpretation of mutation frequency. Because structural alterations typically alter the copy number of a locus, they also have an impact on the relative frequency of any single nucleotide variant (SNV) mutations at that locus. Thus, to assign SNVs to clones, it is necessary to 'correct' for the impact of copy number alteration (CNA), to turn the allelic frequency of an SNV into a clone frequency. In theory, this is straightforward: the cellular abundance of any individual mutation is simply a product of its frequency and copy number. However, if the allelic copy number is incorrectly inferred, then the SNVs in that CNA will be scaled to the wrong frequency, and so potentially erroneously appear as a new clone. In a tumour composed of 50% cancer cells, the difference in frequency of an SNV present on 1 of 3 copies versus 1 of 4 copies is only about 3%, which is a level of accuracy that is rarely achievable with moderate-depth sequencing (~100X). Moreover, errors can stem from in the initial inference of the copy number of the locus. Consequently, errors in the allelic copy number inference propagate to produce an erroneous clone phylogenetic tree, and give a misleading picture of the clonal structure of a tumour. Considering only SNVs located in diploid regions, and exploiting the hitchhiking principle [34] helps, but in a highly aneuploid **[G]** genome risks discarding the majority of SNVs for downstream evolutionary analysis, and potentially important driver mutations that define a clone may be missed. There remains a need for higher resolution data (>100x depth at whole-genome resolution) and improved clonal decomposition methods that effectively handle error propagation from copy number assignments. Emerging long-read sequencing technology also offers the hope of circumventing this issue, as long reads intrinsically 'phase' mutations and so directly reveal their allelic identity.

Single-cell sequencing

Single-cell sequencing is an exciting emerging alternative to bulk sequencing for exploring tumour evolution [35-39] [36,38,40-42]. In theory, sequencing individual cells removes the time bias inherent to bulk sequencing as all the genetic mutations within the sequenced cell, irrespective of when the mutations arose, should be detectable. Clonal identity also becomes evident, removing the need for allelic copy-number correction. However, calling SNVs in single-cell sequencing remains challenging, as it is not possible to distinguish mutational signal from noise by aggregating information across multiple sequencing reads (as is the case for bulk sequencing). Combining information from multiple cells addresses this issue[43]. By contrast, CNAs can be reliably identified [44] but because the background CNA rate is still not well understood [45] drawing inferences about temporal evolutionary dynamics from these data is not straightforward. Nevertheless, single-cell sequencing offers a powerful route to learning how CNAs accrue (since sequencing individual cells means that some newly born cells can be analysed prior to the effects of selection, informing the 'background' CNA mutation rate[45]). Single-cell sequencing of cells from a large cancer risks sequencing many cells that are evolutionary 'dead ends' and would not contribute to the future disease progression. Simply sequencing large numbers of cells would abrogate this issue, and moreover, gives a direct means to detect and characterize negative selection[46], which cannot be identified by bulk sequencing. We expect single-cell sequencing to become the tool of choice in the future as sequencing costs continue to fall.

Detecting selection

Clonal selection drives cancer evolution, and so naturally there is much interest in identifying the cause of a clone's selective advantage, but detecting selection comes with several challenges (**Figure 2**). There are two broad approaches for detecting selection: (1) clone frequency methods and (2) sequence-based methods, and the two approaches are often used concurrently.

Using clone frequency to detect selection

Broadly, frequency-based methods detect selection by looking for lineages that are more abundant than is expected in the absence of selection under neutral evolution. Frequency-based methods have been developed that use the clone size distribution, also referred to as the site frequency spectrum (SFS), which can be measured by the VAF distribution, after correction for tumour purity and copy number. The appeal of this approach is that the shape of the clone size distribution under neutral evolution in a well-mixed population is well known [47 48 6 49 7]. Multi-region sampling can also be used to measure clone frequency: selection for an ancestral clone causes it to have a disproportionate number of offspring in the phylogenetic tree constructed from these data[50]. Hybrid methods that simultaneously consider the VAF distributions from multi-region sampling also exist [51].

Longitudinal sampling of clone abundance provides a particularly powerful method to detect selection: clones that grow disproportionately faster than others are likely under selection [52]. However, longitudinal tissue collection and temporal analyses of solid tumours is rendered challenging by accessibility to tumour tissue. In due course, as sequencing technologies improve and costs decline, we anticipate circulating free tumour DNA analyses will help to circumvent some of these challenges[53,54].

Frequency-based methods are limited by the power to detect deviations from the null neutral model [8]. Weak selection (e.g. a relative selective advantage ~1%) causes only slow and slight shifts in clone frequency that may go undetected in moderate-depth sequencing. The spatial architecture of a tumour presents a complication too — selection is invisible if all the samples are taken within the selected clone. Moreover, frequency-based methods can only detect selection if the selected clone is sampled when expanding; once the clone has taken over the population, the new (fitter) population of tumour cells is homogenous with respect to the selective alteration, and so the within-tumour evolution reverts to neutral. If clones are very strongly selected then clonal expansion to fixation **[G]** in the tumour will be very quick and so unlikely to be detected. In this case, dense longitudinal sampling is necessary to accurately detect selection. There are therefore multiple caveats in inferring a neutral evolution model from single low sequencing depth samples.

Using mutational patterns to detect selection

Alternative methods use the burden and type of mutations across the genome to detect selection (collectively we refer to these as "mutational pattern" methods). These methods exploit the fact that selection causes an over-representation of the mutations that increase fitness, but do not necessarily increase the frequency of neutral mutations. Indeed, statistical tools to identify cancer driver mutations across tumours work by considering the frequency at which a gene is found to be mutated across cancers compared to background expectation [55]. The dN/dS ratio — the ratio of non-synonymous mutations (N mutations) to synonymous mutations (S mutations) normalized by their respective likelihood of occurrence — is a popular sequence-based method for detecting selection. The logic of the method is that non-synonymous mutations will tend to experience selection, whereas synonymous mutations will be evolutionary neutral, and so positive selection will cause an over-representation of NS mutations (dN/dS>1) whereas negative selection will cause an under-representation of NS mutations (dN/dS<1) [56]. Driver mutations have positive dN/dS values [23] and newly refined powerful methods for dN/dS calculation have been developed specifically for cancer data[13,57].

For the dN/dS method to work, there has to be a sufficient number of mutations under selection in the gene or locus to cause a significant deviation of the ratio away from 1. Hence, a minimum mutation burden is required to calculate the ratio at all, and so the method is challenging to apply to individual genes that will carry no more than a few mutations in a single tumour. Whereas dN/dS methods are suitable for looking across cohorts, they are hard to apply to individual tumour evolutionary dynamics. Population demographics also influence the dN/dS ratio in a complex manner and potentially confound its interpretation [58,59]. Moreover, there is evidence that certain synonymous mutations can be under purifying selection which can impact dN/dS estimates and inference of selection.

Stochasticity versus determinism

In small populations, both in cancer and species, stochasticity can dominate the evolution of even strongly selected mutations[3], but a large clone in a large population can behave more deterministically[60]. The threshold between stochastic and becoming deterministic is inversely proportional to the selective advantage of the mutant [52,61 22]. This ‘evolutionary rule’ about the transition from stochasticity to determinism has implications for the predictability of cancer evolution: small, stochastically evolving clones have unpredictable evolution, whereas large clones evolve more predictably. In other words, we are likely to be able to accurately predict the evolution of clones that have already grown large enough to be detected, but an accurate prediction about the emergence of specific minor clones will be more challenging.

Chromosome instability in cancer evolution

CIN and clonal fitness

Alterations in copy number affect a greater proportion of the cancer genome than any other mutations [62], and can act as “hopeful monsters”[63], offering potentially high adaptive advantage to evolving cancers. They result from CIN, a consequence of ongoing errors in chromosome segregation during mitosis and errors of DNA replication and repair[64 65]. The end result is aneuploidy (an unbalanced chromosome complement) involving entire chromosomes (whole-chromosome aneuploidy) or parts of chromosomes (partial or segmental aneuploidy). Aneuploidy can also occur independently of CIN if a single event of chromosome missegregation leads to expansion of the aneuploid clone followed by clonal stasis, that is, without detectable ongoing CIN. Such tumours are homogeneously or clonally aneuploid, whereas tumours with ongoing CIN are heterogeneously or subclonally aneuploid [66,67]. In addition, aneuploidy can result from single catastrophic events, termed chromoplexy **[G]** (if affecting multiple chromosomes) or chromothripsis **[G]** (affecting 1–2 chromosomes), the relevance of which has become increasingly evident across different cancer types [68]. Whatever the mechanism, aneuploidy can alter the somatic copy number, and therefore expression, of many genes at the same time. Although the background alteration rate varies substantially across chromosomes [45] it does not account for evidence of recurrent chromosomal level or arm level aberrations in tumours[69], which can best be explained by selection (positive or purifying). Location of tumour suppressor genes and oncogenes re-capitulates the patterns of aneuploidy observed across different cancers [70,71] also shows the adaptive potential provided by CIN. In a mouse model of acute lymphoblastic leukaemia and hepatocellular carcinoma, induction of CIN in T cells and hepatocytes resulted in tumour-specific patterns of chromosome copy alterations, suggesting that selective pressure is tissue context-dependent[72]. CIN can also provide means of disease escape following curative treatment with surgery or disease control with targeted therapy. Induction of CIN in the KRAS model of lung cancer resulted in rapid relapse, with recurrent tumours showing high levels of aneuploidy [73], with emergent independence from the original oncogenic stimulus. In chronic myeloid leukaemia patients who developed resistance to BCR-ABL targeting imatinib developed additional chromosomal alterations[74].

Some effects of CIN are independent of gene-specific alterations, including reduced proliferation, proteotoxic stress, metabolic changes, upregulation of the stress response and further genome instability. The latter in particular has a profound impact as aneuploid cells continue to create more genetic diversity [75,76]. The fact that aneuploidy (or CIN) can be both detrimental and advantageous highlights the importance of determining the selective landscape. This is well illustrated in yeast where aneuploidy provides a fitness advantage under severe environmental conditions, acting as “a first evolutionary line of defence”[77], but does not persist upon reversion to normal conditions. In a systematic study of the oncogenic potential of aneuploidy in mouse embryonic fibroblasts (MEFs), trisomy failed to induce transformation under any conditions and the cells grew poorly compared to matched euploid cells, consistent with a fitness penalty. However, during long-term growth, triploid cells acquired other aneuploidies that conferred improved fitness [78]. The authors suggest that low levels of aneuploidy may be tumour-protective, but that the genome-destabilizing effects of aneuploidy are tumour-promoting under certain growth conditions. Thus, the rare growth-promoting aneuploidies expand and rise to clonal levels, whilst growth-inhibitory aneuploidies are selected against. Consistent with this notion, aneuploid cells grew better than euploid cells under conditions of environmental stress such as hypoxia and chemotherapy [79]. Addition of a single chromosome increased the tolerance to environmental stresses and was not chromosome-specific, suggesting that overexpression of particular genes is not the only contributor to adaptive potential.

CIN and metastases

Complex processes of metastatic spread, which require a multitude of cellular phenotypes could be well served by the karyotypic and phenotypic heterogeneity generated by CIN. Comparative studies of matched primary tumour–metastasis pairs have reported enrichment for aneuploidy in metastatic lesions from prostate, pancreatic, breast and colon cancers (reviewed in [80]). Through a detailed clonal resolution of matched clear cell renal cell cancer (ccRCC) primary and metastatic tumours, we recently reported that a critical difference between tumour clones that are metastasis-competent compared to those that fail to metastasize is the degree of aneuploidy and chromosome complexity (measured by fluorescence-activated cell sorting (FACS) and weighted genome instability index [81] [82]). Furthermore, we observed that specific somatic CNAs, loss of 9p and loss of 14q, were highly enriched within the metastasizing clones, reflecting active selection. We found no evidence of selection for the smaller scale mutations such as SNVs[82]. Beyond altering the expression of many genes simultaneously potential mechanism by which chromosomal alterations contribute to metastasis include the induction of mesenchymal transition through changes in expression of intercellular junction proteins[83], activation of cGAS–stimulator of interferon genes (STING) pathway by cytosolic DNA from chromosome missegregation[84] [85,86] , and immune evasion [87].

CIN and clinical outcomes

The role of CIN in cancer evolution and progression is evidenced by its association with poor clinical outcomes in a number of retrospective studies [88,89]. More recently, analyses in a prospective cohort of early stage non-small cell lung cancer (NSCLC) evolution (TRACERx-Lung study) showed that CIN confers an increased risk of recurrence and death independently of known predictive markers [26]. In TRACERx-Renal, a similarly prospective study of clear cell renal cell carcinoma (ccRCC), increase in aneuploidy was associated with shorter progression-free and overall survival[25]. Intriguingly, the level of CIN has a bearing on its overall impact on prognosis. In a pan-cancer analysis of >2,000 samples, only moderate levels of CIN (>25% and <75%) were associated with decreased survival, concordant with previous studies showing that excessive levels of CIN confer an improved prognosis [90,91] [92]. These observations are consistent with a fitness cost of CIN, with the selective advantage of karyoptypic heterogeneity negated by excessive levels of aneuploidy.

CIN is also linked to resistance to anti-cancer treatment, including chemotherapy [93,94], and CTLA4 and PD1 immune checkpoint inhibitors [87] [95]. In NSCLC, CIN can lead to subclonal loss of heterozygosity (LOH) in the genes encoding the human leukocyte antigen (HLA) [96], with pervasive evidence of positive selection for this event in tumours. In this context, HLA LOH facilitates accumulation of subclonal neoantigens, and further clonal evolution [96]. In ccRCC, we observed increased rates of HLA LOH in primary tumour subclones that were selected in metastatic sites, highlighting again the role of immune evasion in metastasis[82].

Evolutionary patterns and patient outcomes

A critical question is whether understanding a tumour's evolutionary trajectory and evolutionary potential can help to predict patient outcomes. In particular, the presence of clonal diversity is expected to provide a rich repertoire of alterations that could be adaptive under selective pressure of therapy, alterations in tumour environment or metastatic colonisation of distant sites. In a prospective study of Barrett's oesophagus, a premalignant condition, progression to adenocarcinoma correlated with clonal diversity independently of other genetic risk factors [97]. Multiple studies have demonstrated the link between subclonal diversification and adverse clinical outcomes in chronic lymphocytic leukaemia [98] [99], head and neck cancer [100] ovarian cancer [101] and across other cancer types [102]. Subclonal diversification of somatic CNA and mutational drivers was associated with adverse prognostic features in ccRCC, and independently associated with reduced progression-free and overall survival [25]. In NSCLC, diversity of SCNAs but not SNVs correlated with the risk of relapse and death [26]. In patients with breast cancer, intratumour heterogeneity of HER2 copy number, detected at single-cell resolution, was associated with shorter survival [103].

However, lack of detectable clonal diversity does not always correlate with improved clinical outcome. In multiple myeloma, detection of neutral evolution dynamics correlated with progression-free and overall survival[106] and associated with the presence of a strong clonal oncogenic driver, which might explain the lack of ongoing selection. It is also increasingly apparent that some tumours acquire multiple and/or strong drivers in a short period of time (punctuated evolution), whereas others show a more steady rate of driver acquisition (gradual evolution) [10,107-109]. The result of punctuated evolution is a rapid clonal sweep and a fairly homogenous tumour mass. In ccRCC, these tumours are characterized by low driver intratumour heterogeneity and high levels of clonal aneuploidy which became fixed early on in tumour evolution. These tumours proliferated faster, disseminated rapidly to many different sites (Figure 3a), and had worse outcome, compared to those characterized by clonal diversity and subclonal aneuploidy[25]. Metastases from rapidly evolving tumours were seeded by the same dominant clone found at the primary site resulting in limited inter-metastatic heterogeneity in untreated patients (Figure 3a). By contrast, tumours with subclonal aneuploidy, evolving in a Darwinian fashion and gradually accumulating driver alterations, grew more slowly and over longer periods of time. In some cases metastases were seeded by multiple clones resulting in inter-metastatic heterogeneity (in untreated patients). In line with this, a mathematical model of metastases formation suggests that the probability of observing inter-metastatic heterogeneity (which results from distinct clones in the primary tumour seeding different metastatic sites) increases when the primary tumour grows slowly[110]. Intriguingly, gradually evolving tumours were also associated with a specific pattern of metastatic progression, termed "oligometastases" [82] (Figure 3b). Oligometastases, defined as a small number of lesions confined to a single site, are conceptualized as an "intermediate state of metastatic capacity" [111,112] with an important clinical implication for directed, potentially curative treatment for such lesions. Reduced metastatic efficiency of clonally diverse tumours could be a result of clonal interference (inter-clonal competition at the primary tumour site) or a reflection of weak clonal drivers, with subclonal driver events providing additional fitness required for metastases.

Pancreatic cancer has traditionally been viewed as following gradual evolution with sequential acquisition of driver events. However, some pancreatic cancers show punctuated equilibrium as the principle evolutionary trajectory, whereby multiple driver events are acquired, sometimes through a single 'catastrophic' event results in complex genomic rearrangements [113]. Consistent with our observations in renal cancer, such evolutionary trajectories result in limited inter-metastatic heterogeneity, as all metastases are seeded by the dominant primary tumour clone [114]. Another example is uveal melanoma, characterized by aggressive though latent liver metastases in a proportion of patients, especially those whose primary tumour harbours *BAP1* mutations. *BAP1* mutations and chromosomal complexity were shown to arise in a short burst early on in tumouregensis[107], implying that metastatic potential can be acquired at the earliest stages of cancer evolution. Similar observations have been made in triple-negative breast cancer [109], while chromoplexy and chromotripsis were shown to fuel rapid evolution in prostate cancer and colorectal cancer [108,115], respectively.

Finally, the temporal order in which mutations are acquired during tumour evolution impacts the clinical phenotype and outcome in myeloproliferative neoplasms[104], ccRCC [25] , NSCLC and breast cancer[105]. These observations are consistent with determinism, and suggest that evolutionary trajectories could potentially be predicted for patient benefit.

The observation of the wide spectrum of evolutionary patterns in cancer begins to reconcile the diverse clinical phenotypes and varied outcomes seen in the clinic. In particular, the occurrence of punctuated genomic evolution highlights the challenge of managing cancers that acquire metastatic competency early, cancers that are 'born to be bad'. Supporting this notion are pre-clinical models which show metastatic dissemination before frank malignancy is detected histologically[116]. These observations are especially relevant for cancer screening approaches. As the latency between the emergence of the invasive clone and metastatic spread can be short the window for early detection could be very limited [117]. Many questions about evolutionary trajectories remain including the environmental conditions which favour gradual evolution (gradual accumulation of driver mutations), or punctuated evolution (large-scale rearrangements of the genome leading to many drivers acquired at once) and how these may be altered for therapeutic benefit.

Origin of the treatment-resistant clone

Resistance to targeted therapies

Targeting oncogenic drivers in both blood and solid malignancies has brought about a remarkable change in the cancer treatment landscape. Notable examples include BCR/ABL translocation in chronic myeloid leukaemia (CML), where the use of imatinib has resulted in 10-year survival rates of ~85% [118]; KIT mutations in gastrointestinal stromal tumours (GISTs), HER2 amplification in breast cancer, EGFR mutations in NSCLC, and BRAF mutations in melanoma. However, with the exception of CML, disease control afforded by targeted agents is fairly short-lived, and treatment rarely results in long-term survival for the patient. Mutational complexity of solid cancers may be a contributing factor to inevitability of resistance, as every additional mutation could provide a pathway to treatment resistance. Accordingly, higher tumour mutational burden (TMB) correlates with shortened benefit from EGFR-tyrosine kinase inhibitor (TKI) in metastatic EGFR-mutant NSCLC [119]. Although resistance mutations can arise de-novo [120], they frequently pre-exist as minor subclones (Figure 4a) [121,122] though the ability to detect them in pre-treatment samples is limited by the breadth of sampling and depth of sequencing. Modelling of tumour growth suggests that detectable metastatic lesions can harbour ten or more resistant subclones [123]. Although there are limitations to these models (reviewed in [124]), the predictions are consistent with the observations in clinical and genomic data. In a recent study of patients with chronic lymphoid leukaemia treated with ibrutinib, resistance was attributable to the emergence of mutations in BTK and/or PLCG2 which were detected with a high-sensitivity method up to 15 months prior to clinical progression, with some patients evolving multiple resistance mutations[125]. Polyclonal treatment resistance has been described in other tumour types, with evidence of parallel expansion of clones harbouring distinct mechanisms of resistance under selective pressure of therapy [126-128]. Upfront evaluation of the resistant clones can also be used to forecast the duration of therapeutic benefit, as recently demonstrated in metastatic colorectal cancer using frequent time-course liquid biopsies and mathematical modelling [129].

Thus, a comprehensive catalogue of resistant mutations could inform appropriate combinatorial strategies, while dynamic monitoring of emerging and resolving alterations can facilitate adaptive treatment strategies. This approach was well illustrated by the example of EGFR inhibition in colorectal cancer and the waxing and waning of the resistant RAS-mutant alleles in the blood in response to treatment initiation and withdrawal [130]. These observations also highlight the issue of fitness penalty associated with resistant mutations: KRAS mutations were detected in cell-free DNA from patients who developed resistance to EGFR inhibition; however, when therapy was withdrawn they remained undetectable, suggesting that they require ongoing therapy for their maintenance and that resistance comes at a cost. The higher the fitness cost, the harder it is for the resistant clone to emerge as modelled in xenografts derived from patients (**[G]** (PDXs) with BRAF-V600E mutant melanoma or NSCLC, who developed resistance to BRAF inhibition. PDXs were exposed to ERK inhibition (downstream of BRAF), which resulted in multiple BRAF-amplified clones being selected and propagated. When BRAF, MEK and ERK inhibition were combined in an intermittent schedule, the fitness disadvantage prevented the emergence of the BRAF-amplified subclones [131]. Finally, clonal complexity may impact the drug target itself. Although frequently clonal by virtue of being founder alterations, drug targets can also be found in tumour subclones. In a recent clinical trial FGFR inhibitor responders harboured a clonal FGFR amplification, whereas non-responders harboured subclonal amplifications [132].

Resistance to immune checkpoint inhibition

Another important development in cancer therapeutics has been the advent of immune checkpoint blockade **[G]**. The efficacy of checkpoint inhibitors (CPIs) is contingent on pre-existing recognition of the tumour by the immune system, through presentation of neoantigens which result from somatic mutations accumulated by the tumour. Accordingly, the best responses are observed in tumours with an abundance of somatic mutations (that is, a high TMB), which increases the likelihood of a potent neoantigen being presented to the immune system. Initially, it was expected that CPIs might circumvent the clonal diversity faced by targeted therapies; however, it has become apparent that clonal evolution has a profound impact on immunotherapy success and failure. Subclonal neoantigens do not stimulate an adequate tumour response, as shown by reduced sensitivity to checkpoint blockade in melanoma and NSCLC tumours that have a significant proportion of subclonal mutations [133]. This pattern has been confirmed across additional tumour types [134]. Neoantigen evolution, or immune-editing, underlies some aspects of acquired resistance to CPIs. Both clonal and subclonal neoantigens loss under selective pressure of CPI treatment have been reported. Clonal neoantigens are lost through deletion of the chromosome region that harbours the alteration, whereas subclonal neoantigens are lost through outgrowth of alternative subclones [135]. Critically, peptides generated from the lost neoantigens elicited clonal T-cell expansion in autologous T-cell cultures, suggesting that they generated functional immune responses [135]. Neoantigen immune editing has also been reported in the context of adoptive transfer of autologous lymphocytes that specifically target proteins encoded by cancer-specific mutations, another area of active clinical development which holds much promise [136]. T-cell recognized neoantigens were selectively lost over time in metastatic melanomas treated by adoptive T-cell transfer [137], accompanied by development of neoantigen-specific T-cell reactivity in tumour-infiltrating lymphocytes, indicating immunediting.

Inactivation of antigen presentation is another important mechanism of acquired CPI resistance. For example, point mutations, deletions or LOH in *B2M*, which encodes an essential component of MHC class I antigen presentation, and in the genes encoding interferon-receptor-associated Janus kinase 1 (JAK1) or JAK2, have all been reported as common mechanisms [138 37]. Just as with the drivers of resistance to targeted therapy, these alterations were selected and expanded under therapy. Vaccine strategies are also vulnerable to these alterations. In a trial of an RNA-based vaccine against a spectrum of cancer mutations, neo-epitope-specific killing was demonstrated in a patient who initially responded, but developed resistance owing to the outgrowth of β2-microglobulin-deficient melanoma cells [139]. Another mechanism of immune evasion occurs through selection of tumour populations where HLA is either mutated or lost. In a recent report of adoptive T cell transfer in a patient with colorectal cancer, profiling of a progressive lesion revealed loss of the chromosome 6 haplotype encoding the HLA allele that recognized the targeted mutant KRAS [140].

Conclusions and perspective

An understanding of the dynamics of cancer evolution might lead to improvement in clinical outcomes, as it enables prognoses to be accurately determined and 'evolution-aware' patient management to be applied. Genomic analysis provides a quantitative measurement of evolutionary dynamics and evolutionary potential. There is tremendous value still to be gleaned from analyses of the rapidly-growing public repository of cancer genomic data; particular insight can be gained from the large sample numbers and the inter-comparison of evolutionary dynamics between cancer types. However, we caution that our inferences are severely restricted by the limitations of single-biopsy, bulk-sequenced data sets. As sequencing costs continue to fall, deeper sequencing will allow more accurate determination of clonal fractions (reducing error on inferences derived from these data) and enable the resolution of smaller clones. Single-cell sequencing technology promises to circumvent much of the complexity of 'bulk' sequencing data, and this maturing technology promises the concurrent measurement of genotypes and phenotypes in individual cells [141], together with a characterization of their in-situ microenvironment [36].

Improving the availability of samples from which to study cancer evolutionary dynamics also presents a bottleneck: we hope initiatives such as our TRACERx [142] and PEACE [143] studies, which provide infrastructure for longitudinal and post-mortem collection of tumour samples, will become more common. Even at a single time-point, these studies provide greater representative tumour sampling relative to single-tumour biopsies, which under-represent tumour bulk, leading to the risk of clonal illusion. Quantitative genomic analysis of 'liquid biopsies' (the analysis of tumour DNA from peripheral blood samples) may overcome this issue and provide an amenable route for minimally-invasive longitudinal disease monitoring as well as predictions on disease course and treatment response [53,129,144-146]. In summary, evolutionary genomics provides an ever-improving lens to reveal the clonal dynamics of cancer and impact patient outcomes.

Box 1. Is cancer a special case of evolution?

Despite major overlaps between evolutionary biology and cancer biology, there are a few aspects of cancer evolution that indicate tumours may be a special case of evolutionary systems. First, tumours are extremely large populations, much larger than most common ecosystems and more akin to bacteria colonies, with populations in the order of 100s of billions of cells. This implies that the total diversity is astounding. Another special feature of cancers is that chromosomal instability, which is central to cancer evolution. Chromosomal instability allows for the generation of true 'hopeful monsters' — grossly altered clones that may be adaptive — a phenomena thought to be very rare in species evolution. Cancer cell plasticity, or phenotypic change that does not require underlying heritable variation, is also a fundamental force that guides tumour adaptation and makes the system rather 'non-Darwinian' in some contexts.

Figure 1. Modes of cancer evolution. Cancers evolve according to Darwinian rules: mutation and selection of beneficial new mutations drives the expansion of subclones, and between and within selected clones, the cellular populations experience neutral drift. Different 'modes' of evolution appear depending on when and how the evolutionary process is sampled.

Figure 2. Challenges in detecting selection. a. Limited sampling in time and space confounds measurement of evolutionary dynamics. (i) Sampling within a clone shows neutral dynamics. (ii) Non-uniform spatial sampling can look like selection when it is absent because of genetic divergence, or *vice versa*. (iii) If driver mutations accrue rarely but exhibit a strong effect, most evolutionary time shows only neutral dynamics. (iv) Selection occurs within a small niche that is below the detection limit, so evolution appears neutral because selected subclones are undetectable. (v) Using frequency/phylogenetic methods, selection can only be detected when a clone boundary is sampled. **b.** Bulk sequencing data has a profound time bias, allowing only the earliest – and so highest frequency – mutations to be detected. As a tumour doubles its cell number, new mutations that arise represent an exponentially smaller fraction of the tumour, and so rapidly fall below detectable frequency. **c.** Error in copy-number assignment propagates and confounds the identification of tumour subclones. Limited depth sequencing (say 100X) causes dispersion in the true VAF of a variant, and true VAF is determined by clonal abundance and underlying copy-number state (coloured shapes on plot). This leads to mutations in different clones, or at different copy-number states, being erroneously misassigned clonal identities (red boxes). The $1/f^2$ tail of low frequency mutations is an inevitable consequence of tumour growth, and further complicates clonal inference on VAF data.

Figure 3. Clonal evolution and metastases. Different modes of evolution in the primary tumour can impact the mode of metastatic progression[25]. Metastatic capacity is associated with increased chromosome complexity[82]. **A**. Tumours that evolve in a punctuated fashion

with early onset of clonal chromosome complexity grow rapidly and metastasise early and widely. Metastases are monophyletic (single dominant clone seeds all the metastatic sites) and monoclonal (single clone seeds single site), and there is limited inter-metastatic heterogeneity. **B**. Tumours that evolve in a branched/Darwinian fashion grow more slowly are composed of distinct subpopulations of cells with differential metastatic capacity and chromosome complexity is acquired late. They can be associated with solitary or oligo-metastases. When they spread to multiple sites they may do so in a polyphyletic fashion (different subclones seed different sites), which may include organ-specific patterns and result in inter-metastatic heterogeneity[110]. If multiple clones seed the same site the metastasis is polyclonal.

Figure 4. Clonal evolution of treatment resistance. A. Resistant mutations can be present in the tumour population before the start of therapy, usually as a minor subclone[123,125]. They may evade detection in the baseline sample if they are present at very low frequency or a restricted to an unsampled region of the tumour. They may be even neutral or deleterious before therapy. Under the selective pressure of therapy, the treatment-sensitive population diminishes leaving the resistant population to expand under positive selection. Multiple subclones bearing distinct resistant mutations can emerge at the same time, indicating parallel evolution of resistance[126-129]. **B**. Treatment resistance can be a result of a de novo mutation which carries a selection advantage under therapy and becomes fixed in the tumour population. In this case resistance takes longer to emerge[120].

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Glossary

Clonal evolution

A process by which genetic and epigenetic alterations create diversity that acts as substrate for natural selection.

Subclone

A populations of cells in the tumor that harbour the same set of genomic alterations

Genetic drift

A stochastic random process that changes subclone frequency

Selection

A non-random process shaped by environmental and tumour properties that changes subclone frequency

Molecular evolution

Evolutionary change at the level of DNA sequence.

Somatic evolution

Accumulation of genomic alterations in somatic cells

Chromosome instability

A type of genomic instability that involves parts of or entire chromosomes.

Mutator phenotype

Increase in mutation rates in cancer

Neutral evolution

Clonal diversity not caused by selection

Phylogenetic tree

A branching diagram showing the hierarchy of clones within the tumour

Clonal sweep

Reduction of diversity due to the fixation of a variant due to strong positive selection.

Punctuated equilibrium

Rapid speciation events with long periods of intervening stasis.

Hopeful monster

The generation of an individual with a grossly-altered genome compared to its ancestor, which may be adaptive. A hopeful monster is the result of punctuated change in the genome.

Passenger mutation
A mutation that has no effect on clone fitness

Driver mutation
A mutation that increases clone fitness

Variant Allele Frequency
Relative frequency of a variant in a tumour sample, expressed as a percentage

Aneuploid
The presence of an abnormal chromosome complement

Fixation
Rise of a variant in frequency in the population to 100%

Chromoplexy
A complex rearrangement of the cancer genome that involves a number of chromosomes

Chromothripsis
A complex rearrangement of the cancer genome that involves a single chromosome

Patient-derived xenografts
A tumour model where the tissue from patient's tumour is implanted in an immunodeficient mouse.

Immune checkpoint blockade
Therapies that target immune checkpoints such as CTLA4 and PD1 which tumours can use to escape anti-tumour immune responses