Trends in Cancer

CellPress

Review

Re-Evaluating Clonal Dominance in Cancer Evolution

Rebecca A. Burrell[1] and Charles Swanton[1,2,*]

Tumours are composed of genetically heterogeneous subclones which may diverge early during tumour growth. However, our strategies for treating and assessing outcome for patients are overwhelmingly based upon the classical linear paradigm for cancer evolution. Increasing numbers of studies are finding that minor subclones can determine clinical disease course, and that temporal and spatial heterogeneity needs to be considered in disease management. In this article we review evidence for cancer clonal heterogeneity, evaluating the importance of tumour subclones and their growth through both Darwinian and neutral evolution. Major shifts in current clinical practice and trial designs, aimed at understanding cancer evolution on a patient-by-patient basis, may be necessary to achieve more successful treatment of heterogeneous metastatic disease.

Tumour Evolution and Defining the 'Dominant Clone'

A Darwinian evolutionary framework for cancer development was proposed in 1976 by Peter Nowell, delineating how tumour progression could arise from sequential development and selection of mutant subpopulations derived from a common progenitor [1].

In the decades that have followed we have gained an increasingly detailed understanding of the complexity of cancer evolution [2]. Rather than uniformly 'sequential selection of more aggressive sublines' [1], cancer evolution often follows a branched trajectory, with divergent subclones evolving simultaneously [3,4]. Both metastasis and therapeutic resistance can be driven by minor and sometimes multiple subclones [5] rather than necessarily by ongoing evolution of an increasingly aggressive dominant clone.

In the current era of increasing personalisation of cancer medicine, spatial separation of subclones and variation in the subclonal composition of tumours over time pose significant challenges for cancer diagnostics and treatment [6]. While one clone may dominate the physical entity of a tumour, minor and often undetectable subclones can dominate the clinical course of cancer [7–9]. There is also emerging evidence that subclones within tumours may cooperate to promote tumour growth, indicating that minor subclones can have functional roles, rather than being byproducts of evolution of the dominant clone.

How then do we define a dominant clone, and is such a definition an accurate or useful portrayal of the complex dynamic behaviour of the subclonal populations constituting a tumour? As we acquire greater understanding of the dynamics of cancer evolution, and of the importance of minor, or subclonal populations to tumour evolution and disease progression, this may shape novel approaches to diagnostics, treatment, and disease monitoring in clinical practice.

Trends

Next-generation sequencing has revealed the complexity of tumour evolution. Cancers are usually composed of multiple genetically related, distinct subclones evolving in parallel. This represents a shift from the traditional paradigm of ongoing evolution of one increasingly aggressive clone.

Evolutionary forces in heterogeneous tumours are complex. There may be both competition and cooperation between subclones. Recent evidence also suggests that neutral evolution may be common across a range of tumours.

Subclonal architecture varies over space and time. Spatial heterogeneity poses a challenge of sampling bias, particularly if individual subclones dominate tumour regions. Circulating tumour DNA enables monitoring of disease burden and drug resistance over time, potentially addressing issues of sampling bias and the requirement for repeated tumour sampling.

[1]Translational Cancer Therapeutics laboratory, Francis Crick Institute, 44 Lincoln's Inn Fields, London WC2A 3LY, UK
[2]University College London (UCL) Cancer Institute and Hospitals, Cancer Research UK (CRUK) Lung Cancer Centre of Excellence, Huntley Street, London WC1E 6DD, UK

*Correspondence:
charles.swanton@crick.ac.uk
(C. Swanton).

Spatial Variation in Clonal Dominance

Next-generation sequencing studies have revealed substantial intratumour genetic heterogeneity (ITH) within tumour lesions, between metastatic and primary tumours, and over time. Mutations identified in tumour cells at all disease sites and time-points conforming to clonal estimates will have likely been present in the last common ancestor cell before genetic divergence of any subclonal populations, and are on the trunk of the evolutionary tree of the tumour (Figure 1). A proportion of these clonal or 'truncal' mutations are likely to have been early events involved in the initial transformation from normal to neoplastic cellular behaviour, while others are passenger mutations present in the founding cell [10]. 'Private' mutations and chromosomal aberrations confined to a particular tumour region or population of cells have now been demonstrated in multiple solid tumour types including, but not limited to, glioblastoma [11], medulloblastoma [12], ovarian [13–15], breast [16–18], renal [3,19], lung [20], oesophageal [21], prostate [22–24], pancreatic [25], and hepatocellular carcinomas [26].

Subclonal populations can be mixed together [17,27,28], or spatially separated within a tumour or between primary and metastatic sites [3,14,19,25] (Figure 1). Multiregion sequencing in clear cell renal cell carcinoma (ccRCC) revealed regional separation of subclones in all cases; only 6/62 regions across eight cases harboured multiple detectable subclones [3,19]. The degree of ITH of single-nucleotide variants (SNVs) increased with the number of biopsies taken, without saturation in most tumours, indicating no single clone physically dominated each tumour [3]. In a multiregion sequencing study of 12 breast cancers, locally confined expansion of subclones was the predominant pattern of heterogeneity [16], although four tumours displayed mixing of subclones, consistent with previous reports resolving multiple subclones from deep sequencing of single biopsies in breast cancer [17,18,29]. Local domination of tumour regions by individual subclones was also seen in lung and oesophageal cancers [20,21].

Further evidence for localised subclonal dominance comes from multifocal breast cancer: within each disease focus, private mutations had high variant allele fractions, indicating a likely founder effect or clonal sweep at each site [16]. Subclonal driver gene mutations were identified in three of four multifocal cancers sequenced [16]. In prostate cancer, there is gross heterogeneity of point mutations and chromosomal aberrations between different foci of multifocal prostate cancer, suggestive of multiclonal disease in some instances [23], in contrast to multifocal breast cancer, where sequencing identified a clonal relationship between disease foci [16]. However, in some cases of prostate cancer, minimal heterogeneity is seen between different sites of disease [30].

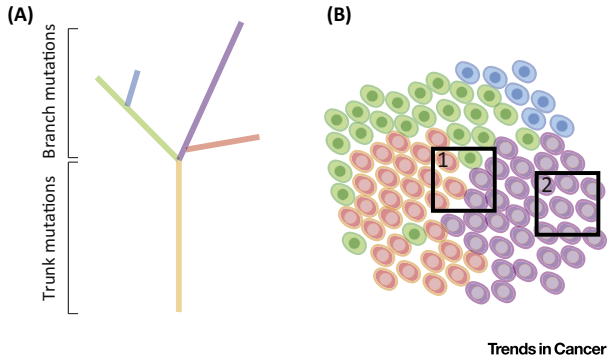

Figure 1. Branched Evolution and Sampling Bias in Solid Tumours. (A) Genetic divergence of subclones during tumour evolution. Mutations present in the last common ancestor will be present on the trunk of the tumour phylogenetic tree. Private mutations in divergent subclones are located on the branches. (B) Sampling bias may confound resolution of the clonal status of mutations. While a biopsy in region 1 would identify three subclones, a biopsy in region 2 would lead to an 'illusion of clonality' owing to the dominance of the tumour area by one subclone.

Such regional subclonal dominance may also be seen in metastasis, with resulting genetic heterogeneity between metastatic sites [19,25]. Distinct mutations in samples from different metastatic niches may reflect variation in selection pressures at these sites–for example, in ovarian cancer, evolutionary clades (groups of more closely related subclones) were noted to correspond to geographical areas from which the samples were taken [15]. Alternatively, heterogeneity between metastases could simply reflect the mutational events present in the founding metastatic clone [25], or the impact of drug therapy. There are now several documented examples of recurrent mutations affecting the same gene or pathway that confer resistance to therapy at different sites of disease [31,32]. It is likely that epigenetic adaptation and stromal factors also contribute to organ-specific adaptation. The significance of private mutations at metastatic sites is difficult to determine–these may be passenger events expanded only by virtue of a founder effect, but could in some cases also reflect a context-dependent driver gene mutation or metastasis-promoting alteration. Together with the fact that the majority of studies aiming to identify metastasis specific mutations have focussed on protein-coding genes, it is also likely that we are underpowered to identify metastasis-specific mutations, which could also affect non-coding regions of the genome or so far undetected epigenetic modifications.

The mechanisms underpinning different subclonal distributions remain unclear. Similarly, whether the spatial organisation of tumour subclones is dynamic or relatively static is not known, although a recent study of HER2 (human epidermal growth factor 2)-positive breast cancer found that changes in spatial organisation of cellular genetic diversity associated with neoadjuvant chemotherapy correlated with poor long-term outcome following adjuvant therapy with trastuzumab [33]. Admixing of subclones within single samples, particularly where histopathology reveals mosaicism [28], could represent co-dependency between clonal populations (discussed further below) or the generation of the private subclonal mutations early on in tumour growth [34]. It remains unclear if spatial separation of subclones therefore impacts upon functional interactions and interdependencies between tumour subclones. In tumour regions dominated by one clone, it is possible that a selective advantage (which may be particular to a given disease site or tumour region) enables a subclone to expand. Recent work has highlighted the role of migration in the expansion of new subclones [35]. Provided there is sufficient cell turnover, a relatively small selective advantage is required for significant expansion of new subclones, allowing them to supersede their ancestors [35].

Notwithstanding this recent insight, where individual subclones are sufficiently expanded to dominate large tumour areas it is unclear whether it is possible for these to be completely superseded by either their own descendants or by the descendants of subclones from other regions. Indeed, micro-mapping of subclones within a cross-section of a hepatocellular carcinoma has revealed that new subclones tend to arise on the peripheries of tumours and expand outwards [26]. Thus, regional separation of subclones within tumours might predicate branching evolution, similar to divergent evolution of subclones at different metastastic sites. Assuming that spatial separation of subclones precludes complete clonal sweeps, and depending on how early in tumour evolution it occurs, it is conceivable that subclonal separation could influence the shape of the overall evolutionary tree, with earlier branching and fewer shared truncal mutations.

Hence, while mutations may be ubiquitously present throughout a tumour, it is unusual for a single clone to dominate the tumour. Instead, tumours are made up of several genetically heterogeneous but related subclones. In some tumours subclones are mixed and may be detected within a single sample, while in others individual tumour regions are dominated by one subclone. Biopsies taken from regions dominated by individual subclones could give an illusion of clonal dominance, where heterogeneous somatic events appear to be clonal when in fact they are late, subclonal events arising on the branches of tumour phylogenies (Figure 1). Without multiple samples, establishing a ‘dominant clone’ is fraught with difficulty owing to varying

dominance of different subclones in different tumour regions or sites of disease. Even with multiple samples, the full extent of ITH may not be resolved [3].

Temporal Variation in Subclonal Architecture

Selection pressures vary through the disease course as well as spatially within a tumour [2]. Heterogeneity may enable wider exploration of the fitness landscape, allowing adaptation in the face of obstacles to tumour growth. Clonal dominance and subclonal architecture may therefore vary substantially over time, most clearly demonstrated in comparing resistant or recurrent disease to pretreatment samples [5,12,31,36].

There are multiple examples of temporal variation in clonal dominance from studies of haematopoietic malignancies, aided by ease of repeat sampling and the representation of multiple subclones within individual blood samples. Dramatic fluctuations in clonal dominance over time have been observed in multiple myeloma, as well as in chronic lymphocytic (CLL), acute lymphoblastic (ALL), and acute myeloid leukaemias (AML). Disease at progression or death was often markedly different from diagnostic samples [7,8,37–40].

Linear and branched evolutionary trajectories have been observed in multiple myeloma and in CLL after treatment [4,37,41]. Interestingly, however, in five of a cohort of 15 cases of multiple myeloma analysed over the course of treatment, stable clonal structure was observed despite overt clinical response [37], implying equal response of all subclones to treatment, with no single dominant clone driving cancer progression (Figure 2). Alternatively, this could indicate that the subclone-defining mutations had minimal differential functional impact (i.e., are neutral), at least in the context of the treatment in question. Nevertheless, there is evidence for selection of subclones bearing driver gene mutations during treatment in CLL and myeloma, with increased subclonal complexity after therapy [4,37,42]. One explanation for this is the relief of interclonal competition through the elimination of one or more subclones, thereby enabling the outgrowth of previously unexpanded subpopulations [4]. In studies of some tumour types, such as melanoma [31], increased subclonal diversity after therapy has been related to polyclonality of drug resistance-conferring mutations. However, it is not clear in all cases whether subclonal driver gene mutations identified in resistant cell populations directly confer resistance to therapy, or whether independent mechanisms of resistance were active simultaneously. The presence of subclonal driver gene mutations in CLL was associated with adverse clinical outcome [4,41].

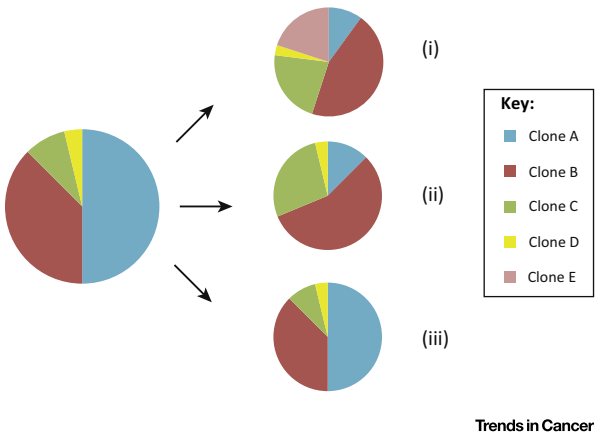

Figure 2. Temporal Evolution of Subclonal Architecture. The subclonal composition of malignancies varies over time, particularly during treatment. (i) Generation of a de novo subclone, or expansion of previously undetected clone; (ii) clonal selection during treatment or over time; (iii) stable complex subclonal architecture.

In solid tumours, evidence for temporal variation is generally derived from comparisons between diagnostic and recurrent samples, or is inferred from comparison of metastatic and primary tumours. For metastases sampled at the same time as primary tumour, the timing of metastasis can only be estimated based on mutational distance, particularly if the metastatic lesions arise from distinct subclones, or from a minor subclone that branches early from the evolutionary tree of a tumour. However, an apparently new subclone emerging during treatment may well have been present but not sampled in the pretreatment specimen [16] (Figure 2). Nevertheless, there are multiple examples of therapeutic resistance/relapse in solid tumours not being driven by a single dominant resistant clone but instead by multiple different subclones, each harbouring distinct resistance-conferring mutations (reviewed in [5]).

The contribution of multiple drug-resistant subclones to temporal heterogeneity has been most clearly demonstrated when new sites of disease are established at relapse [31,43]. It has been estimated that most radiographically detected lesions harbour at least 10 resistant subclones [44]. In a case of melanoma, branched evolution was identified following treatment, with heterogeneity of both resistance-conferring mutations and other SNVs between sites of disease progression [31]. In another study, after adjuvant therapy, recurrent gliomas were found to be commonly derived from minor clones that were ancestral to those dominating the surgically excised primary tumour, with frequent branching evolution between excision and recurrence [36]. Similarly, in a medulloblastoma, recurrence was driven by clonal selection of a pre-existing minor clone present at diagnosis [12] (Figure 3). In three cases of high-grade serous ovarian cancer, repeated sampling of malignant ascites pre- and post-treatment revealed complex fluctuations in clonal architecture in one case, with relatively stable clonal architecture in the other two cases, similar to observations in multiple myeloma [13,37]. Stable clonal architecture despite initial response to treatment could be explained by non-genetic factors such as cancer cell plasticity that enable adaptation and tumour cell persistence, or by drug-resistant cancer stem cells able to repopulate the tumour [45,46].

The challenges of both repeated biopsies and sampling bias to monitor tumour clonal evolution over time in solid tumours may be partly circumvented by sampling circulating tumour DNA (ctDNA), cell-free DNA that is shed from tumour cells [47]. A recent study of colorectal cancers found that, in 8% of tumours, *KRAS/NRAS/BRAF* mutations were identified in ctDNA that were not found in corresponding tissue samples, implying more representative sampling of all tumour sites with ctDNA [48]. The ability to use ctDNA to monitor tumour progression is advancing rapidly, with multiple studies documenting the emergence of mutations conferring resistance to

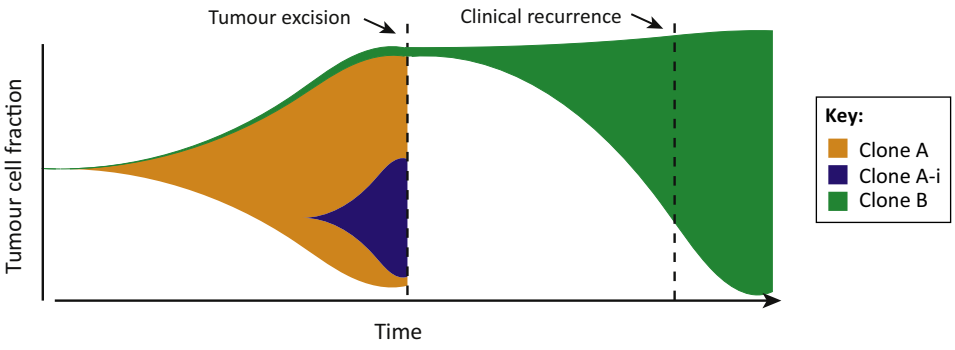

Figure 3. Early Divergence of Recurrent Disease. Recent reports have described the early divergence of subclones that drive disease recurrence [12,36]. In this schematic, before tumour excision clone A was the dominant clone. Subclone A-i (blue) is a nested descendent of clone A. Clone B (green) is a minor clone, which diverged early from clone A, and was barely detectable in the excised sample. Clone B constitutes the disease at clinical recurrence.

therapy in ctDNA samples [47–52]. In many instances, more than one resistance-conferring mutation is detected at disease progression [5]. Serial sampling of ctDNA in a patient with metastatic breast cancer revealed subclonal private mutations that corresponded to differential responses at different sites of disease [52]. However, a significant hurdle for clinical use of this technology is variation in the amount of ctDNA shed by different cancers and by different stages of cancer; for example, fewer than 50% of brain, renal, and prostate cancers (all known to be highly heterogeneous) had detectable ctDNA [47]. Notwithstanding this challenge, ctDNA represents a promising avenue for monitoring heterogeneous and multisite solid tumours over time and in response to treatment [52].

Insights into Tumour Biology from the Study of ITH

The revelation of the extent of ITH and the complexity of cancer evolution from next-generation sequencing studies suggests challenges to personalised cancer medicine, and to current thinking on how to improve cancer therapy. However, it has also given fresh insights into tumour biology, from modes of evolution to novel mutational processes operating at different stages in the disease course [29,53,54]. Sequencing multiple biopsies over time and space, and deep sequencing of individual biopsies, have significantly impacted on our understanding of how cancers evolve. Divergence of subclones, including of metastatic clones [16,19,25] or clones contributing to disease relapse [7,36], can occur early, with evolution following a branched trajectory in a significant proportion of malignancies [55]. Reconstructing tumour phylogenies reveals the relative timing of driver mutations, highlighting key events in the initiation and progression of different malignancies. Studies of large cohorts of tumours can reveal whether mutations in given genes/pathways are more likely to be clonal (and hence potentially early or founder events) or subclonal (late events) [53].

Through delineating the timing of different mutations based on their clonal status, it has been possible to mine sequencing data to identify mutational processes operating at different stages during tumour evolution [17,29,53,54,56]. Such approaches have led to the identification of a role for the APOBEC cytidine deaminase family in the generation of point mutations in a wide range of tumours [29,53,57,58]. Deregulated function of these enzymes leads to increased deamination of cytosine to uracil, resulting in an increased frequency of C-to-T transition mutations, as well as clustering of point mutations (reviewed in [59]). In lung adenocarcinomas exposed to tobacco carcinogens, the relative contribution of smoking to the mutational burden [60,61] decreases over time, while conversely the contribution of APOBEC-related mutagenesis increases [20], being enriched in branched mutations. APOBEC mutagenesis has also been implicated in the genesis of late subclonal mutations in other tumour types including bladder cancer, oestrogen receptor-negative breast cancer, and head and neck squamous cell carcinomas [53].

In ccRCC, 73–75% of driver gene mutations were subclonal or branched mutations [3], while mutations in *VHL* together with loss of chromosome 3p were always clonal events, implicating these changes as key founding events in ccRCC. Conversely, in breast cancer many common breast cancer genes, including *PIK3CA, TP53, PTEN*, and *BRCA2*, can be mutated either ubiquitously or found only in subclones (i.e., can occur early or late), with no strict mutation order being evident [16]. In multiregion sampled lung cancers, high-confidence mutations in category 1 driver genes were more often clonal compared to non-driver genes [20]. Across single samples from a larger dataset of lung cancers, driver gene mutations were enriched for clonal status, suggesting that common mutations such as *EGFR* mutations occur early in lung cancer development, although larger scale multiregion sequencing studies will be necessary to fully define such patterns [20]. Notwithstanding the potential caveat of incorrectly assigned clonal status with only single samples available, mutations in genes in specific pathways were found to be more likely clonal than subclonal–in particular, mutations in RAS–MEK pathways and genes associated with cyclin-dependent kinases were more likely to have clonal status [53].

In oesophageal cancer, there was a trend for high-confidence driver events to be situated on the trunks of the phylogenetic trees. Approximately one quarter of such events were located on branches, including potentially targetable *PIK3CA* mutations [21]. However, *TP53* inactivation was fully clonal and was always accompanied by copy-neutral loss of heterozygosity (LOH), highlighting the importance of P53 inactivation as an early event. All tumour regions analysed showed evidence of genome doubling, consistent with previous reports documenting a key role for this event in the transition from premalignant Barrett's oesophagus to malignant oesophageal adenocarcinoma [62]. Genome doubling has been implicated in rapid genomic evolution and chromosomal instability across a range of cancers [63,64] and, consistent with this, high frequencies of chromosomal aberrations were seen across all oesophageal cancer regions sampled [21]. Chromosomal amplifications were significantly more likely to be clonal than chromosome gains or losses, suggestive of being early events in oesophageal cancer development [21].

Delineating the subclonal architecture of heterogeneous tumours has also revealed the occurrence of multiple distinct mutations in the same genes/pathways across different subclones of the same tumour, termed parallel evolution [3,16,19,21,53]. Analysing single biopsies from 2694 tumours across nine cancer types, 30 cases were identified in which multiple subclonal mutations were identified in the same cancer gene or in separate genes with functional overlap, leading to simultaneous disruption of the same genetic pathway within different subclones in the same tumour [6]. In breast cancer, multiregion sequencing has identified recurrent mutations in multiple genes including driver genes *PTEN*, *FGFR2*, *TP53*, and *RUNX1* [16]. For *TP53, PTEN*, and *RUNX1*, the recurrent mutations represented the second hit on a tumour-suppressor gene, with the first hit being on the trunk of the phylogenetic tree. This has some similarities with parallel evolution in ccRCC [3,19]. Loss of 3p is an early event, together with *VHL* inactivation, that was uniformly found on the trunk of tumour phylogenetic trees in a set of 10 ccRCCs [3,19]. Recurrent mutations occurring in parallel in different branches of the phylogenetic tree were observed in three other tumour-suppressor genes also encoded on 3p: *SETD2, PBRM1* (can be truncal or branched), and *BAP1*, as well as recurrent mutations in *PTEN* and *PIK3CA* [3,19]. Therefore, early events in tumour evolution (Figure 4) might prime the tumour for later somatic events. This is supported by evidence that similar clonal expansion patterns emerge in replicate murine xenografts of the same starting human tumour cell population, suggesting that genomic features can predict future tumour evolutionary trajectories [65].

Recurrent alterations within tumours implicate specific pathways as key in cancer development, and suggest evolutionary constraints despite gross heterogeneity. Nevertheless, the functional importance of subclonal driver gene mutations is not always clear. Similarly, mechanisms of resistance to targeted therapies appear to converge upon a relatively constrained range of mutations and aberrations, often in multiple parallel clones within the same malignancy [31,49,66]. Such recurrent resistance mechanisms may therefore be anticipated and potentially exploited therapeutically.

Neutral Versus Darwinian Evolution in Cancer Growth

Recent work has called into question the ubiquity of Darwinian evolution and selective forces in cancer growth [26,67]. In 'neutral evolution', all the mutations responsible for expansion of the tumour are present in the founding cell, and any mutational events that occur subsequently have minimal or no impact on tumour expansion (i.e., they are neutral). Where there is an observed linear correlation between allelic frequency and the overall number of subclonal mutations, this is consistent with neutral evolution [67]. Analysis of sequencing data from single biopsies over 14 tumour types from The Cancer Genome Atlas found that growth of a little over 31% of tumours appeared to fit neutral evolutionary dynamics [67]. Such redundancy or neutrality of subclonal mutations may be consistent with estimates that only three sequential mutations are

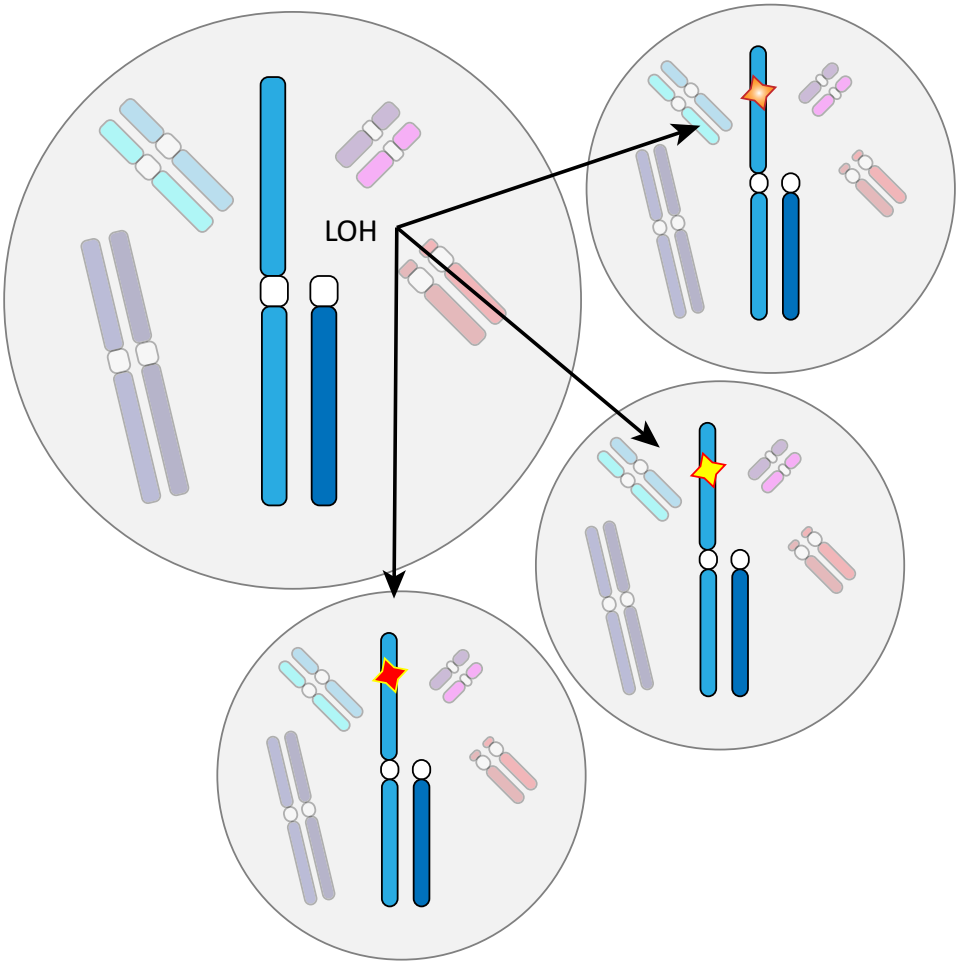

Figure 4. Early Clonal Genetic Changes May Prime Tumour Evolution. Studies in clear cell renal cell carcinoma and breast cancer have revealed recurrent mutations in the same driver genes [3,16,19]. These recurrent mutations often occur as the second 'hit', with the first hit arising as loss of heterozygosity (LOH) on the trunk of the evolutionary tree of the tumour. Thus, founder clonal events may prime the tumour for alterations in particular genes or pathways.

necessary to drive the cancer phenotype in lung and colon cancers [68]. Interestingly, neutral evolution appeared to be more common in some tumour types (e.g., stomach, lung, bladder) than in others (e.g., renal, melanoma, pancreatic, glioblastoma). The accurate classification of subclonal and clonal mutations is clearly fundamental to this approach. Low subclonal mutation burden also influences the likelihood of a tumour being ascribed as neutral because the likelihood of detecting a significant correlation between allelic frequency and cumulative mutation load is reduced. With this in mind, it is interesting that a smaller fraction of microsatellite-unstable tumours (with high mutational loads) were found to fit the neutral model compared to microsatellite-stable tumours in both colon and gastric cancers [67]. This may be due to an increased likelihood of selection against a detrimental mutation.

Of particular note, some putative driver gene mutations were found to have occurred during neutral phases of tumour growth (i.e. they were not subject to positive selection), pointing towards context-dependence of the 'driving' effect of these mutations [67]. This suggests that we need to consider not only presence or absence of subclonal driver mutations but

also the evidence for selection (such as recurrence of mutations affecting the same gene/pathway and the ratio of non-synonymous to synonymous mutations [3,53]) and the context in which they occur, for example during therapy or metastasis. It remains to be determined whether tumours can go through both neutral and Darwinian phases of growth and, if so, how these modes of evolution might relate to different phases of tumour growth and progression. Further studies of multiregion sampled tumours, including metastatic sites, will be imperative. It will also be important to study tumours over time to dissect out the influence of treatment upon modes of tumours growth. Because neutral evolution may theoretically generate greater intratumour genetic heterogeneity than growth under selection [26], neutral growth may facilitate adaptation after the onset of selection (for example initiation of treatment).

Clonal Cooperation in Heterogeneous Tumours

It is conventional to think of interactions between tumour cell populations in terms of competition [1]. However, as our understanding of cancer heterogeneity increases, so too does our appreciation of interactions between not only cancer cells and stromal or immune cells but also between subclonal populations of cancer cells. There is an increasing body of evidence, thus far largely from experimental models, indicating that subclonal populations may cooperate to further tumour growth [69].

Such cooperation can take the form of direct crosstalk between populations of cells [70], paracrine signalling [71,72], or occur through micro-environmental remodelling by one clone that benefits another [73,74]. There is evidence that paracrine signalling between distinct subclonal populations can facilitate metastasis in a mouse model of small cell lung cancer [75,76]. Similarly, colorectal cancer cells sensitive to EGFR blockade can be rendered resistant via secretable factors from resistant subclones [77].

It is also possible that tumour formation, maintenance, and progression might require multiple subpopulations of cells. In mouse models, oligoclonal clusters of circulating tumour cells (CTCs) were associated with a significantly (23- to 50-fold) increased metastatic potential, and oligoclonal CTC clusters were associated with adverse outcome in patient cohorts–reduced progression-free survival in breast cancer patients and reduced overall survival in prostate cancer [78]. A correlation between size of tumour and heterogeneity was observed in triple-negative breast cancer [16]. This may reflect the difficulty of completing a clonal sweep in a larger tumour, or alternatively could indicate that heterogeneous tumours have a growth advantage and grow larger. This latter model is supported by mouse models, in which heterogeneous xenograft tumours derived from a breast cancer cell line were larger and more aggressive than monoclonal xenografts [71].

Given numerous reports of subclones being confined to certain areas [3,15,16], it will be interesting to investigate whether the spatial organisation of subclones is incidental or whether it confers important properties upon the tumour cells and their interactions. The emerging evidence for cooperation between subclones to further both tumour growth and other phenotypes such as metastasis and drug resistance suggests that the notion that tumour progression is driven by the ongoing evolution of a single increasingly aggressive dominant clone may, in some cases, be a simplification.

Therapy Guided by Assessment of Clonal Status

There is now a significant body of evidence documenting the presence of subclonal populations in tumours, including heterogeneity of targetable or 'actionable' mutations. Of 50 breast cancers subjected to high-coverage sequencing of a targeted set of 360 known cancer genes, 13 tumours harboured potentially targetable mutations that were present subclonally [16].

Targeting a dominant, but not a ubiquitous mutation, may run the risk of relieving interclonal competition between expanded subclones and aggressive minor subclones. After targeting the major clone, minor subclones can outgrow and constitute disease relapse or progression [4,33,37,41,42]. Whether such heterogeneity of targetable mutations is as prevalent between metastatic sites (which are particularly relevant for therapy given that the primary tumour is often excised) remains to be fully evaluated. If metastatic spread represents a bottleneck, such that metastases are commonly monophyletic (descended from a common ancestor), then it is possible that driver events might be more uniform between metastases than within the primary tumour [30]. Autopsy studies will be necessary to answer this important question.

Nevertheless, possible approaches to treating heterogeneous tumours include: (i) targeting ubiquitous mutations with combination therapy to pre-empt common resistance mechanisms. (ii) Targeting high-risk subclones. (iii) Adaptive therapy aiming to maintain drug-sensitive populations and stable disease, rather than to eradicate disease. (iv) Immune approaches targeting clonal tumour neoantigens and mutational burden

Considering clonal status and specifically targeting clonal (or truncal) mutations has been proposed as a strategy to treat heterogeneous tumours [53,79]. This approach is supported by the observation that some targets of established therapies with clinical efficacy (for example *EGFR* mutations in lung cancer [20]) are identified most often on the trunks of tumour phylogenetic trees. As we further elucidate the evolution of different malignancies, mutations that are most commonly ubiquitous rather than subclonal will become apparent.

A challenge to this approach is the presence of subclonal resistant populations. There is evidence for selection of subclones harbouring resistance mutations during therapy, with low-frequency resistant clones being identified before treatment [5,43,80–82]. More commonly, however, the resistant population is not detected in the pretreatment sample. This is likely to reflect sampling bias compounded by the potential low frequency of these populations of cells [31,47,50,66,83], but can also reflect de novo evolution of a resistant subclone. Recurrent tumours after surgery may arise from clones that were divergent from the primary tumour, and therefore may have a distinct catalogue of mutations [12,36]. Evaluating mutational signatures in recurrent high-grade glioma revealed a likely contribution of temozolomide therapy to the genesis of driver mutations identified at recurrence, which were implicated in the transformation to aggressive glioblastoma multiforme [36]. There is also evidence for a direct impact of cytotoxic therapies upon the mutational spectra in AML [9] and oesophageal tumours [21].

Resistance, in particular to targeted therapies, appears to recurrently affect the same genes, gene families, or pathways–for example, the emergence of *KRAS* mutations in response to EGFR blockade in colorectal cancers [49,66] and *NRAS/KRAS* mutations in response to BRAF V600E blockade in melanoma [31]. Therefore, one strategy to mitigate the effects of subclonal resistance mutations is combination therapy that aims to simultaneously target the resistant subpopulations. Such efforts are in clinical trials, for example in melanoma by combining BRAF and MEK inhibitors [84]. Modelling approaches have suggested that dual combination therapy may provide durable long-term responses if there are no single mutations conferring cross-resistance [85], although triple therapy may be necessary in patients with high disease burden. Approximately 60% of *EGFR* mutant tumours that developed resistance to anti-EGFR therapy through the *T790M* gatekeeper mutation responded to treatment with rociletinib (a third-generation EGFR inhibitor which is active against *T790M* as well as *EGFR* activating mutations) [86]. Studying resistance mechanisms to rociletinib revealed that *T790M* wild-type clones (which still bear an *EGFR* activating mutation) emerge, while others show *EGFR* amplification. This is a

clear demonstration of how heterogeneity can overcome strategies aimed at targeting individual resistance mechanisms [86,87].

An important consideration in treating heterogeneous tumours is that of clinical versus clonal dominance. Targeting an aggressive subclone may still yield some clinical benefit: for example, targeted treatment of HER2 and the oestrogen receptor in breast cancer even when there is evidence for ITH [88–90]. In a recent study of paired brain metastases and primary tumours of mixed types, 53% of cases harboured actionable mutations that were exclusively seen in the brain metastases, and not in the primary tumours [91]. In a scenario such as this, it is not clear what the optimal therapeutic strategy would be. Ideally the oncologist would target the bulk of disease by targeting clonal mutations. However, there could be a role for specifically targeting brain metastasis-enriched somatic events given they are associated with significant morbidity and mortality, perhaps at the expense of targeting disease elsewhere in the body as effectively. This study also underscores the need for sampling techniques that assess all sites of disease, given such profound heterogeneity between primary and metastatic sites [91]. Risks of targeting subclonal populations, however, in addition to unchecked growth of the rest of the tumour cell population, would include paradoxical stimulation of growth in cells not sensitive to the target, such as the effect of BRAF V600E inhibitors upon the growth of wild-type cells [42,92].

Serial profiling of ctDNA from blood of colorectal cancer patients can be used to dynamically track tumour evolution through treatment with anti-EGFR agents [48]. Importantly, mutant *KRAS* allele percentage declined after cessation of therapy, indicating a dependence of the resistant cell population on EGFR blockade, or at least a fitness deficit with respect to other clones once blockade is withdrawn. Patients with a decline in the *KRAS*-mutant fraction in their ctDNA after initial treatment achieved partial responses or tumour stabilisation in response to retreatment with anti-EGFR agents, and *KRAS*-mutant allele frequency increased once again during second-line therapy. In xenograft models of malignant melanomas treated with vemurafenib, regression of resistant tumours is observed upon withdrawal of the drug, indicating dependence of the tumour upon the drug for ongoing proliferation [93]. This may in part reflect the paradoxical stimulation of *BRAF* wild-type cells by vemurafenib, as discussed above [42,92].

Data suggestive of fitness deficits of resistant cells upon drug withdrawal lend support to adaptive intermittent therapy approaches to reduce the outgrowth of resistant cells to attempt to maintain the tumour in a treatable state for as long as possible [94]. However, while decline of the *KRAS*-mutant population with withdrawal of therapy in colorectal cancer suggests that sensitive disease might be maintained for longer, a balance must of course be struck between retaining sensitivity and unchecked re-growth of the *KRAS* wild-type population during treatment cessation. Fitness deficits after drug cessation may be rare, or clinically insignificant. Further preclinical and clinical studies will be necessary to more fully evaluate the effects of withdrawing treatment or of intermittent treatment relative to standard dosing regimens and rational combination therapy [85,94]

Finally, there has been a recent surge of interest in cancer immunotherapy, with the success of immune checkpoint inhibitors in clinical trials in some malignancies [95,96]. Neoantigens generated by mutational processes active in tumours may be a key component of the recognition of tumour cells by the immune system [97]. Studies have reported that neoantigen and overall mutational burden influences sensitivity to immune checkpoint blockade in NSCLC and melanoma [98–100]. This has led to suggestions that combining immunotherapies with mutagenic chemo- and radiotherapy may show efficacy [101]. Further research will be necessary to evaluate the impact that the clonal status of neoantigens (whether the neoantigen is clonal or branched) has upon response to immunotherapy [102].

Concluding Remarks

An acknowledgement of tumor evolution needs to be built into clinical trial design, with assessment of clonal status rather than simply assessing the presence or absence of a mutation, together with minimally-invasive longitudinal sampling to assess clonal evolution in real-time and identify high-risk subclones that might themselves be targetable to mitigate relapse. If evolutionary constraints exist, such that the next step in a cancer evolution can be anticipated, this may aid the development of treatment strategies for progressive disease (see Outstanding Questions). However, the elucidation of such intricacies of cancer evolution will require large longitudinal studies, with serial tissue and serum samples. Autopsies in the context of longitudinal clinical studies will be fundamental to better understand and define the behaviour of lethal subclones [103]. Increasing ability to detect mutations in serum samples lends hope to more personalised therapy based on dynamic sampling of disease over time [47,48,50,52].

Given numerous examples of substantial clonal evolution during treatment, appropriate clinical trials will need to build-in prospective tissue-harvesting protocols as standard so as to expedite exploration of the mechanisms of resistance and tumour evolution during treatment if we are to harness the benefit of the treatment armoury we have available. A greater understanding of functional interactions between different subclones, as well as heterotypic interactions with stromal and immune cells, will be imperative and may reveal novel therapeutic strategies [69,78].

Outstanding Questions

What are the best strategies for overcoming sampling bias in spatially-heterogeneous tumours? There are limitations to both multiregion sampling and to the use of circulating tumour DNA. Longitudinal studies analysing both in parallel will reveal the potential clinical utility of both approaches.

Can we overcome the challenge of emergent drug-resistant subclones? Delineating common tumour evolutionary pathways and common resistance mechanisms may enable better prediction of the most likely 'next evolutionary move' of the cancer.

How common are functional interactions between subclones within tumours, and how can we disrupt these interactions to halt tumour progression and drug resistance? Current insight into clonal cooperation is largely from murine models–how can we better study this phenomenon in human cancers?

How common is neutral evolution in cancer? Are there both neutral and Darwinian phases of growth during tumour development and progression?

Can adaptive approaches to cancer therapy successfully restrain disease progression and maintain tumours in a 'treatable' state for longer?

How does intratumour genetic heterogeneity interplay with the tumour immune response?

Acknowledgments

The authors would like to thank Dr Nicky McGranahan for helpful discussions. C.S. is a senior CRUK clinical research fellow and is funded by CRUK (TRACERx), the CRUK Lung Cancer Centre of Excellence, Stand Up 2 Cancer (SU2C), the Rosetrees Trust, NovoNordisk Foundation (ID 16584), the European Commission Framework Programme 7 (projects PREDICT and RESPONSIFY, ID: 259303), the Prostate Cancer Foundation, the Breast Cancer Research Foundation, the European Research Council (THESEUS) and the National Institute for Health Research, the UCL Hospitals Biomedical Research Centre, and the CRUK UCL Experimental Cancer Medicine Centre.

References

1. Nowell, P.C. (1976) The clonal evolution of tumor cell populations. *Science* 194, 23–28
2. Greaves, M. and Maley, C.C. (2012) Clonal evolution in cancer. *Nature* 481, 306–313
3. Gerlinger, M. *et al.* (2014) Genomic architecture and evolution of clear cell renal cell carcinomas defined by multiregion sequencing. *Nat. Genet.* 46, 225–233
4. Landau, D.A. *et al.* (2013) Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell* 152, 714–726
5. Burrell, R.A. and Swanton, C. (2014) Tumour heterogeneity and the evolution of polyclonal drug resistance. *Mol. Oncol.* 8, 1095–1111
6. McGranahan, N. and Swanton, C. (2015) Biological and therapeutic impact of intratumor heterogeneity in cancer evolution. *Cancer Cell* 27, 15–26
7. Mullighan, C.G. *et al.* (2008) Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. *Science* 322, 1377–1380
8. Keats, J.J. *et al.* (2012) Clonal competition with alternating dominance in multiple myeloma. *Blood* 120, 1067–1076
9. Ding, L. *et al.* (2012) Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature* 481, 506–510
10. Welch, J.S. *et al.* (2012) The origin and evolution of mutations in acute myeloid leukemia. *Cell* 150, 264–278
11. Sottoriva, A. *et al.* (2013) Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proc. Natl. Acad. Sci. U. S. A.* 110, 4009–4014
12. Morrissy, A.S. *et al.* (2016) Divergent clonal selection dominates medulloblastoma at recurrence. *Nature* 529, 351–357
13. Castellarin, M. *et al.* (2013) Clonal evolution of high-grade serous ovarian carcinoma from primary to recurrent disease. *J. Pathol.* 229, 515–524
14. Bashashati, A. *et al.* (2013) Distinct evolutionary trajectories of primary high-grade serous ovarian cancers revealed through spatial mutational profiling. *J. Pathol.* 231, 21–34
15. Schwarz, R.F. *et al.* (2015) Spatial and temporal heterogeneity in high-grade serous ovarian cancer: a phylogenetic analysis. *PLoS Med.* 12, e1001789
16. Yates, L.R. *et al.* (2015) Subclonal diversification of primary breast cancer revealed by multiregion sequencing. *Nat. Med.* 21, 751–759
17. Nik-Zainal, S. *et al.* (2012) The life history of 21 breast cancers. *Cell* 149, 994–1007
18. Shah, S.P. *et al.* (2012) The clonal and mutational evolution spectrum of primary triple-negative breast cancers. *Nature* 486, 395–399
19. Gerlinger, M. *et al.* (2012) Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N. Engl. J Med.* 366, 883–892
20. de Bruin, E.C. *et al.* (2014) Spatial and temporal diversity in genomic instability processes defines lung cancer evolution. *Science* 346, 251–256
21. Murugaesu, N. *et al.* (2015) Tracking the genomic evolution of esophageal adenocarcinoma through neoadjuvant chemotherapy. *Cancer Discov.* 5, 821–831
22. Cooper, C.S. *et al.* (2015) Analysis of the genetic phylogeny of multifocal prostate cancer identifies multiple independent clonal expansions in neoplastic and morphologically normal prostate tissue. *Nat. Genet.* 47, 367–372
23. Boutros, P.C. *et al.* (2015) Spatial genomic heterogeneity within localized, multifocal prostate cancer. *Nat. Genet.* 47, 736–745
24. Brocks, D. *et al.* (2014) Intratumor DNA methylation heterogeneity reflects clonal evolution in aggressive prostate cancer. *Cell Rep.* 8, 798–806

25. Yachida, S. *et al.* (2010) Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* 467, 1114–1117
26. Ling, S. *et al.* (2015) Extremely high genetic diversity in a single tumor points to prevalence of non-Darwinian cell evolution. *Proc. Natl. Acad. Sci. U. S. A.* 112, E6496–E6505
27. Szerlip, N.J. *et al.* (2012) Intratumoral heterogeneity of receptor tyrosine kinases EGFR and PDGFRA amplification in glioblastoma defines subpopulations with distinct growth factor response. *Proc. Natl. Acad. Sci. U. S. A.* 109, 3041–3046
28. Snuderl, M. *et al.* (2011) Mosaic amplification of multiple receptor tyrosine kinase genes in glioblastoma. *Cancer Cell* 20, 810–817
29. Nik-Zainal, S. *et al.* (2012) Mutational processes molding the genomes of 21 breast cancers. *Cell* 149, 979–993
30. Kumar, A. *et al.* (2016) Substantial interindividual and limited intraindividual genomic diversity among tumors from men with metastatic prostate cancer. *Nat. Med.* 22, 369–378
31. Shi, H. *et al.* (2014) Acquired resistance and clonal evolution in melanoma during BRAF inhibitor therapy. *Cancer Discov.* 4, 80–93
32. Juric, D. *et al.* (2015) Convergent loss of PTEN leads to clinical resistance to a PI(3)Kalpha inhibitor. *Nature* 518, 240–244
33. Janiszewska, M. *et al.* (2015) In situ single-cell analysis identifies heterogeneity for PIK3CA mutation and HER2 amplification in HER2-positive breast cancer. *Nat. Genet.* 47, 1212–1219
34. Sottoriva, A. *et al.* (2015) A Big Bang model of human colorectal tumor growth. *Nat. Genet.* 47, 209–216
35. Waclaw, B. *et al.* (2015) A spatial model predicts that dispersal and cell turnover limit intratumour heterogeneity. *Nature* 525, 261–264
36. Johnson, B.E. *et al.* (2014) Mutational analysis reveals the origin and therapy-driven evolution of recurrent glioma. *Science* 343, 189–193
37. Bolli, N. *et al.* (2014) Heterogeneity of genomic evolution and mutational profiles in multiple myeloma. *Nat. Commun.* 5, 2997
38. Kim, T. *et al.* (2015) Clonal dynamics in a single AML case tracked for 9 years reveals the complexity of leukemia progression. *Leukemia* 30, 295–302
39. Lawrence, M.S. *et al.* (2013) Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* 499, 214–218
40. Anderson, K. *et al.* (2011) Genetic variegation of clonal architecture and propagating cells in leukaemia. *Nature* 469, 356–361
41. Landau, D.A. *et al.* (2015) Mutations driving CLL and their evolution in progression and relapse. *Nature* 526, 525–530
42. Lohr, J.G. *et al.* (2014) Widespread genetic heterogeneity in multiple myeloma: implications for targeted therapy. *Cancer Cell* 25, 91–101
43. Van Allen, E.M. *et al.* (2014) The genetic landscape of clinical resistance to RAF inhibition in metastatic melanoma. *Cancer Discov.* 4, 94–109
44. Bozic, I. and Nowak, M.A. (2014) Timing and heterogeneity of mutations associated with drug resistance in metastatic cancers. *Proc. Natl. Acad. Sci. U. S. A.* 111, 15964–15968
45. Sharma, S.V. *et al.* (2010) A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* 141, 69–80
46. Greaves, M. (2015) Evolutionary determinants of cancer. *Cancer Discov.* 5, 806–820
47. Bettegowda, C. *et al.* (2014) Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci. Transl. Med.* 6, 224ra24
48. Siravegna, G. *et al.* (2015) Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. *Nat. Med.* 21, 827
49. Misale, S. *et al.* (2012) Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature* 486, 532–536
50. Misale, S. *et al.* (2014) Blockade of EGFR and MEK intercepts heterogeneous mechanisms of acquired resistance to anti-EGFR therapies in colorectal cancer. *Sci. Transl. Med.* 6, 224ra26
51. Bardelli, A. *et al.* (2013) Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer. *Cancer Discov.* 3, 658–673
52. Murtaza, M. *et al.* (2015) Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. *Nat. Commun.* 6, 8760
53. McGranahan, N. *et al.* (2015) Clonal status of actionable driver events and the timing of mutational processes in cancer evolution. *Sci. Transl. Med.* 7, 283ra54
54. Stephens, P.J. *et al.* (2012) The landscape of cancer genes and mutational processes in breast cancer. *Nature* 486, 400–404
55. Gerlinger, M. *et al.* (2014) Cancer: evolution within a lifetime. *Annu. Rev. Genet.* 48, 215–236
56. Pleasance, E.D. *et al.* (2010) A small-cell lung cancer genome with complex signatures of tobacco exposure. *Nature* 463, 184–190
57. Roberts, S.A. *et al.* (2013) An APOBEC cytidine deaminase mutagenesis pattern is widespread in human cancers. *Nat. Genet.* 45, 970–976
58. Taylor, B.J. *et al.* (2013) DNA deaminases induce break-associated mutation showers with implication of APOBEC3B and 3A in breast cancer kataegis. *Elife* 2, e00534
59. Swanton, C. *et al.* (2015) APOBEC enzymes: mutagenic fuel for cancer evolution and heterogeneity. *Cancer Discov.* 5, 704–712
60. Govindan, R. *et al.* (2012) Genomic landscape of non-small cell lung cancer in smokers and never-smokers. *Cell* 150, 1121–1134
61. Imielinski, M. *et al.* (2012) Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. *Cell* 150, 1107–1120
62. Galipeau, P.C. *et al.* (1996) 17p (p53) allelic losses, 4N (G2/tetraploid) populations, and progression to aneuploidy in Barrett's esophagus. *Proc. Natl. Acad. Sci. U. S. A.* 93, 7081–7084
63. Carter, S.L. *et al.* (2012) Absolute quantification of somatic DNA alterations in human cancer. *Nat. Biotechnol.* 30, 413–421
64. Dewhurst, S.M. *et al.* (2014) Tolerance of whole-genome doubling propagates chromosomal instability and accelerates cancer genome evolution. *Cancer Discov.* 4, 175–185
65. Eirew, P. *et al.* (2015) Dynamics of genomic clones in breast cancer patient xenografts at single-cell resolution. *Nature* 518, 422–426
66. Diaz, L.A. *et al.* (2012) The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* 486, 537–540
67. Williams, M.J. *et al.* (2016) Identification of neutral tumor evolution across cancer types. *Nat. Genet.* 48, 238–244
68. Tomasetti, C. *et al.* (2015) Only three driver gene mutations are required for the development of lung and colorectal cancers. *Proc. Natl. Acad. Sci. U. S. A.* 112, 118–123
69. Tabassum, D.P. and Polyak, K. (2015) Tumorigenesis: it takes a village. *Nat. Rev. Cancer* 15, 473–483
70. Cleary, A.S. *et al.* (2014) Tumour cell heterogeneity maintained by cooperating subclones in Wnt-driven mammary cancers. *Nature* 508, 113–117
71. Marusyk, A. *et al.* (2014) Non-cell-autonomous driving of tumour growth supports sub-clonal heterogeneity. *Nature* 514, 54–58
72. Inda, M.M. *et al.* (2010) Tumor heterogeneity is an active process maintained by a mutant EGFR-induced cytokine circuit in glioblastoma. *Genes Dev.* 24, 1731–1745
73. Chapman, A. *et al.* (2014) Heterogeneous tumor subpopulations cooperate to drive invasion. *Cell Rep.* 8, 688–695
74. Mateo, F. *et al.* (2014) SPARC mediates metastatic cooperation between CSC and non-CSC prostate cancer cell subpopulations. *Mol. Cancer* 13, 237
75. Calbo, J. *et al.* (2011) A functional role for tumor cell heterogeneity in a mouse model of small cell lung cancer. *Cancer Cell* 19, 244–256
76. Kwon, M.C. *et al.* (2015) Paracrine signaling between tumor subclones of mouse SCLC: a critical role of ETS transcription factor Pea3 in facilitating metastasis. *Genes Dev.* 29, 1587–1592
77. Hobor, S. *et al.* (2014) TGFalpha and amphiregulin paracrine network promotes resistance to EGFR blockade in colorectal cancer cells. *Clin. Cancer Res.* 20, 6429–6438

78. Aceto, N. *et al.* (2014) Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. *Cell* 158, 1110–1122
79. Yap, T.A. *et al.* (2012) Intratumor heterogeneity: seeing the wood for the trees. *Sci. Transl. Med.* 4, 127ps10
80. Inukai, M. *et al.* (2006) Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer. *Cancer Res.* 66, 7854–7858
81. Maheswaran, S. *et al.* (2008) Detection of mutations in EGFR in circulating lung-cancer cells. *N. Engl. J. Med.* 359, 366–377
82. Turke, A.B. *et al.* (2010) Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. *Cancer Cell* 17, 77–88
83. Hata, A.N. *et al.* (2016) Tumor cells can follow distinct evolutionary paths to become resistant to epidermal growth factor receptor inhibition. *Nat. Med.* 22, 262–269
84. Richman, J. *et al.* (2015) BRAF and MEK inhibition for the treatment of advanced BRAF mutant melanoma. *Expert Opin. Pharmacother.* 16, 1285–1297
85. Bozic, I. *et al.* (2013) Evolutionary dynamics of cancer in response to targeted combination therapy. *Elife* 2, e00747
86. Sequist, L.V. *et al.* (2015) Rociletinib in EGFR-mutated non-small-cell lung cancer. *N. Engl. J. Med.* 372, 1700–1709
87. Piotrowska, Z. *et al.* (2015) Heterogeneity underlies the emergence of EGFRT790 wild-type clones following treatment of T790M-positive cancers with a third-generation EGFR inhibitor. *Cancer Discov.* 5, 713–722
88. Hammond, M.E. *et al.* (2010) American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *J. Clin. Oncol.* 28, 2784–2795
89. Early Breast Cancer Trialists' Collaborative, G. *et al.* (2011) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet* 378, 771–784
90. Burrell, R.A. *et al.* (2010) Targeting chromosomal instability and tumour heterogeneity in HER2-positive breast cancer. *J. Cell Biochem.* 111, 782–790
91. Brastianos, P.K. *et al.* (2015) Genomic characterization of brain metastases reveals branched evolution and potential therapeutic targets. *Cancer Discov.* 5, 1164–1177
92. Poulikakos, P.I. *et al.* (2010) RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature* 464, 427–430
93. Das Thakur, M. *et al.* (2013) Modelling vemurafenib resistance in melanoma reveals a strategy to forestall drug resistance. *Nature* 494, 251–255
94. Enriquez-Navas, P.M. *et al.* (2015) Application of evolutionary principles to cancer therapy. *Cancer Res.* 75, 4675–4680
95. Brahmer, J.R. *et al.* (2012) Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N. Engl. J. Med.* 366, 2455–2465
96. Brahmer, J. *et al.* (2015) Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *N. Engl. J. Med.* 373, 123–135
97. Schumacher, T.N. and Schreiber, R.D. (2015) Neoantigens in cancer immunotherapy. *Science* 348, 69–74
98. Rizvi, N.A. *et al.* (2015) Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 348, 124–128
99. Snyder, A. *et al.* (2014) Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N. Engl. J. Med.* 371, 2189–2199
100. Van Allen, E.M. *et al.* (2015) Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science* 350, 207–211
101. Melero, I. *et al.* (2015) Evolving synergistic combinations of targeted immunotherapies to combat cancer. *Nat. Rev. Cancer* 15, 457–472
102. McGranahan, N. *et al.* (2016) Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science* 351, 1463–1469
103. Jamal-Hanjani, M. *et al.* (2014) Tracking genomic cancer evolution for precision medicine: the lung TRACERx study. *PLoS Biol.* 12, e1001906