Tumour heterogeneity and resistance to cancer therapies

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Abstract | Cancer is a dynamic disease. During the course of disease, cancers generally become more heterogeneous. As a result of this heterogeneity, the bulk tumour might include a diverse collection of cells harbouring distinct molecular signatures with differential levels of sensitivity to treatment. This heterogeneity might result in a non-uniform distribution of genetically distinct tumour-cell subpopulations across and within disease sites (spatial heterogeneity) or temporal variations in the molecular makeup of cancer cells (temporal heterogeneity). Heterogeneity provides the fuel for resistance; therefore, an accurate assessment of tumour heterogeneity is essential for the development of effective therapies. Multiregion sequencing, single-cell sequencing, analysis of autopsy samples, and longitudinal analysis of liquid biopsy samples are all emerging technologies with considerable potential to dissect the complex clonal architecture of cancers. In this Review, we discuss the driving forces behind intratumoural heterogeneity and the current approaches used to combat this heterogeneity and its consequences. We also explore how clinical assessments of tumour heterogeneity might facilitate the development of more-effective personalized therapies.

Advances in molecular diagnostics and sequencing technologies have been instrumental in uncovering the intricate genetic makeup of many cancers. The process of conversion from a nonmalignant to a malignant cell is now understood to occur largely through the sequential acquisition of alterations that lead to enhanced cellular proliferation, evasion of growth suppression and cell death signals, induction of angiogenesis, and, ultimately, activation of programmes leading to tissue invasion and metastasis[1,2]. The often stochastic nature of cancer initiation reinforces the notion that the development and progression of cancer does not follow a fixed course, but should rather be viewed as an integrated destabilization of key cellular processes[2]. Even after malignant transformation, a cancer remains dynamic and continues to evolve. This ongoing evolution might ultimately generate a molecularly heterogeneous bulk tumour consisting of cancer cells harbouring distinct molecular signatures with differential levels of sensitivity to anticancer therapies. This heterogeneity can result from genetic, transcriptomic, epigenetic, and/or phenotypic changes, although this Review will primarily focus on genetic heterogeneity.

At the population level, tumour heterogeneity can be broadly divided into intertumoural and intratumoural heterogeneity. Intertumoural heterogeneity, which refers to heterogeneity between patients harbouring tumours of the same histological type, has long been recognized and is believed to result from patient-specific factors including germline genetic variations, differences in somatic mutation profile, and environmental factors. The advent of genomic medicine has increased our appreciation of intratumoural heterogeneity, which refers to heterogeneity among the tumour cells of a single patient. Intratumoural heterogeneity can manifest as spatial heterogeneity, which describes the uneven distribution of genetically diverse tumour subpopulations across different disease sites or within a single disease site or tumour, and as temporal heterogeneity, a term applied to the dynamic variations in the genetic diversity of an individual tumour over time (FIG. 1).

Insights into the genomic landscape of some cancers, such as non-small-cell lung cancer (NSCLC), have fuelled a shift in the treatment paradigm towards the use of personalized, or genotype-guided approaches[3,4]. This strategy is most successful for the treatment of cancers that are dependent on key genetic alterations, which are often referred to as 'oncogenic drivers', as such vulnerabilities can be therapeutically exploited using molecularly targeted agents. Despite dramatic initial responses, however, almost all cancers develop resistance to targeted therapies. This observation lends support to the current understanding of cancer as a dynamic, rather than molecularly stagnant, disease. Indeed, the findings of multiple studies

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Key points

- Genomic instability fosters genetic diversity by providing the raw material for the generation of tumour heterogeneity
- Tumours with high levels of intratumoural heterogeneity might predispose patients to inferior clinical outcomes
- Under therapeutic selective pressure, resistance to treatment can emerge as a result of the expansion of pre-existing subclonal populations or from the evolution of drug-tolerant cells
- Serial characterization of genetic variants in plasma samples has the potential to provide information on spatial and temporal heterogeneity on a scale that cannot easily be achieved through analyses of tumour biopsy samples alone
- Multiregion sampling, research autopsies, and single-cell sequencing are all emerging informative platforms that have the potential to enable decoding of complex clonal relationships at a high level of resolution
- Combinatorial approaches that pair therapies targeting the predominant, drug-sensitive population of clones in addition to the various subsets of drug-resistant and drug-tolerant cells seem likely to induce the most-durable responses

across a diverse range of cancer types suggest that intratumoural heterogeneity drives the evolution of cancers and fosters drug resistance[5–9]. Thus, a comprehensive understanding of tumour dynamics is essential for the development of effective and durable therapeutic strategies. In this Review, we will discuss the driving forces behind intratumoural heterogeneity, the spectrum of intratumoural heterogeneity in solid tumours, the contribution of intratumoural heterogeneity to drug resistance, and, finally, how an understanding of intratumoural heterogeneity can inform the design of rational antineoplastic strategies, particularly in the setting of cancers with an established oncogenic driver.

Causes of intratumoural heterogeneity

Genomic instability

Genomic instability, which can range in magnitude from single-base substitutions to whole-genome doublings, is critical to the development and progression of many cancers[2,10]. Such instability might result from exposure to exogenous mutagens (such as UV radiation or tobacco smoke) and aberrations in endogenous processes (such as DNA replication and/or repair errors, or oxidative stress)[11,12]. For example, microsatellite instability (MSI), owing to deficiencies in DNA mismatch repair (MMR), drives neoplastic transformation and substantially increases the somatic mutation burden of the subset of colorectal cancers (CRCs) and other cancers with this distinctive phenotype[13]. Studies involving large-scale genome sequencing have enabled the identification of characteristic genetic signatures associated with some of these mutagenic processes. For example, smoking-related lung cancers are enriched with C > A transversions, and, similarly, MMR-deficient CRCs are prone to C > T transitions[14–16]. Furthermore, although not contributing to baseline genomic instability, exposure to chemotherapy might also increase the mutational spectrum of a tumour and create genomic instability[17–19].

According to certain theories, tumorigenesis is reliant on an elevated spontaneous mutation rate[20,21]. This hypothesis has not been definitively proven and might not apply to all cancers, although data from multiple studies demonstrate that cancers often co-opt endogenous homeostatic processes in order to increase the overall mutational burden. For example, DNA cytosine deamination, resulting from upregulation of DNA dC→dU-editing enzyme APOBEC3B, has been shown to contribute to mutagenesis in approximately half of all human cancers[22–24]. The APOBEC mutational signature, which is characterized by the presence of C>T and C>G mutations at TpC sites, is particularly enriched in the later stages of tumour development and becomes more prevalent after exposure to cytotoxic chemotherapy[24–26]. High levels of APOBEC3B expression portend a poorer outcome among patients with certain cancers[27,28]. For example, a whole-exome sequencing study involving analysis of approximately 1,500 breast cancer samples revealed an association between high levels of APOBEC3B expression and inferior disease-free survival and overall survival outcomes in patients with oestrogen receptor-positive breast cancer[28]. Furthermore, APOBEC mutagenesis can lead to mutations in genes involved in treatment resistance; therefore, successful inhibition of these enzymes might reduce genetic instability and thus improve patient outcomes[24]. Notably, in some tumours, including brain tumours, genomic instability results from chromosome-level changes that lead to gains or losses of whole-genome segments, rather than point mutations[10]. This process, termed chromosomal instability, arises from segregation errors that occur during cell division, which might promote genetic diversity by upsetting the balance between activation of oncogenes and tumour suppressors[29].

The clonal evolution/selection hypothesis

Regardless of the source, genomic instability fosters genetic diversity by providing the raw material for the generation of tumour heterogeneity. In addition to a larger role in creating copy-number imbalances, dynamic chromosomal instability can lead to the non-uniform loss of chromosomal segments harbouring specific genetic alterations and can indirectly contribute to mutational heterogeneity across different tumour regions[6]. Evidence of ongoing genomic instability within certain regions of individual tumours and across different metastatic sites, in addition to the existence of these events at the subclonal level, suggests that increased levels of genomic instability promote the emergence of more-competitive subclones[6,30,31]. Excessive levels of genomic instability can, however, have adverse effects on cancer cell survival and fitness[32,33].

Tumours are likely to require more than just genomic instability alone in order to maintain heterogeneity. Indeed, genomic instability might co-operate with other factors to promote the development of tumour heterogeneity and tumour progression. Various models have been proposed to explain how clonal diversity is generated and maintained, although the majority of contemporary studies continue to use the clonal evolution and/or selection framework model. This model, initially introduced by Peter Nowell in 1976 (REF. 34), is based on the hypothesis that tumour initiation occurs

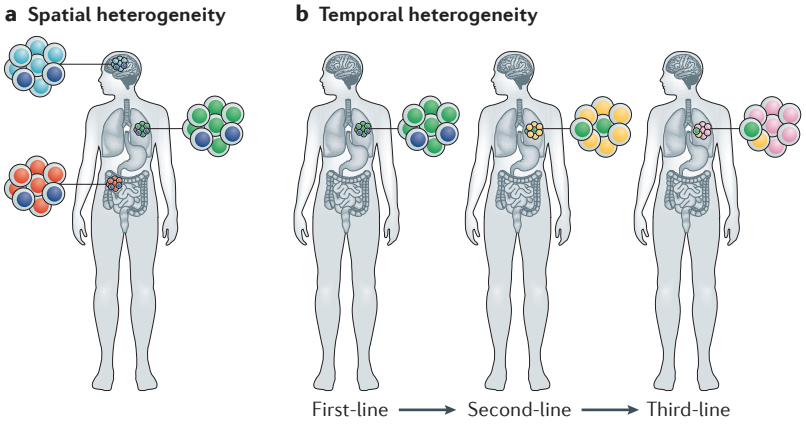

Figure 1 | **A conceptual framework for distinguishing between spatial and temporal intratumoural heterogeneity. a** | Spatial heterogeneity denotes an uneven distribution of cancer subclones across different regions of the primary tumour and/or metastatic sites. **b** | Temporal heterogeneity refers to variations in the molecular makeup of a single lesion over time, either as a result of natural progression of the tumour or as a result of exposure to selective pressures created by clinical interventions. Colours denote the presence of subclones with different genetic features.

in a stochastic manner, beginning with an induced change in a previously nonmalignant cell that confers a selective growth advantage and leads to neoplastic proliferation. Subsequently, the genomic instability of the expanding tumour population creates additional genetic diversity that is subjected to evolutionary selection pressures, resulting in the sequential emergence of increasingly genetically abnormal and heterogeneous subpopulations[34]. In this model of tumour evolution, linear evolution describes evolution owing to the successive acquisition of mutations that confer a growth and/or survival advantage, with sequential clones harbouring these advantageous mutations outcompeting ancestral clones. Alternatively, branching evolution denotes the emergence and divergent propagation of multiple subclonal tumour cell populations that share a common ancestor (FIG. 2). Branched evolution enables a greater level of opportunity to create a more heterogeneous tumour[35–38]. Many solid tumours adopt a branched pattern of evolution, whereas a linear pattern is invoked in phylogenetic depictions of certain haematological malignancies[31,35–41]. Interestingly, the assumption that clonal subpopulations must always be in competition has been challenged by the results of studies showing that cooperation between distinctly different subclones might be necessary for tumour propagation in cancers with non-cell-autonomous initiating events and that metastatic sites can be seeded polyclonally[41–44].

The spectrum of tumour heterogeneity

Spatial heterogeneity

Cancer is characterized by the ability to ignore growth-suppression signals, invade local tissues, and metastasize to distant organs[1,2]. The molecular makeup of cancer cells that predominate at different sites can be distinct, owing to the variable influences of microenvironment-related factors and site-specific stressors. Owing to selective pressures that lead to further genetic evolution, heterogeneity might exist even among the cells present within the parent tumour. Collectively, the uneven distribution of genetically diverse tumour subpopulations across different sites, and sometimes within a tumour in a single anatomical location, is termed spatial heterogeneity. In the following sections, we will discuss the current understanding of spatial heterogeneity in solid malignancies.

Heterogeneity at a single disease site. Primary tumours can contain multiple geographically separated, molecularly distinct cellular subpopulations. This spatial heterogeneity can result in an uneven distribution of key molecular alterations across different regions of the tumour. Alternatively, spatial heterogeneity in the primary tumour might manifest as the ubiquitous presence of key molecular driver alterations, with an unequal distribution of additional molecular alterations. In support of the former hypothesis, the findings of several studies have revealed the clonal dominance of potentially actionable driver mutations within individual regions of breast and oesophageal cancers, and clear-cell renal-cell carcinoma (RCC)[38,45,46]. In support of the latter hypothesis, an analysis of 41 multiregion biopsy samples from eight melanomas revealed a uniform distribution of key molecular drivers, including mutations in *BRAF* and *NRAS*, with a heterogeneous distribution of additional somatic mutations[47]. These examples are not provided to imply that these are mutually exclusive competing theories. Rather, the pattern of spatial heterogeneity observed is more likely reflective of the specific evolutionary context within which the individual tumour arose, such that both patterns might be represented.

Multiregion sampling, which describes biopsy sampling of multiple regions within a single lesion, is an informative investigational strategy that improves the ability to determine the extent of spatial heterogeneity within an individual tumour[6,38,45,47]. For example, in a study by Jamal-Hanjani and colleagues[6], sampling of 327 tumour regions from 100 early stage NSCLCs revealed a substantial level of intratumoural heterogeneity, with a median of 30% of somatic mutations identified as subclonal. Notably, the authors reported that if fewer regions had been sampled, approximately 76% of subclonal mutations would have been misrepresented as clonal[6]. Very few studies have used multiregion sampling; therefore, the prognostic implications of heterogeneity within contiguous tumour regions remain under investigation. One study involving multiregion sampling of melanomas demonstrated that many of the unevenly distributed passenger mutations are not expressed[47]. This heterogeneous gene-expression pattern affects the utility of gene-expression signatures, as markers associated with a good prognosis and different markers associated with a poor prognosis might be present in geographically distinct regions within the same tumour[47,48].

Interestingly, in the previously mentioned study by Jamal-Hanjani *et al.*[6], more-extensive copy-number heterogeneity (defined as >48% subclonal copy-number alterations) was associated with worse clinical

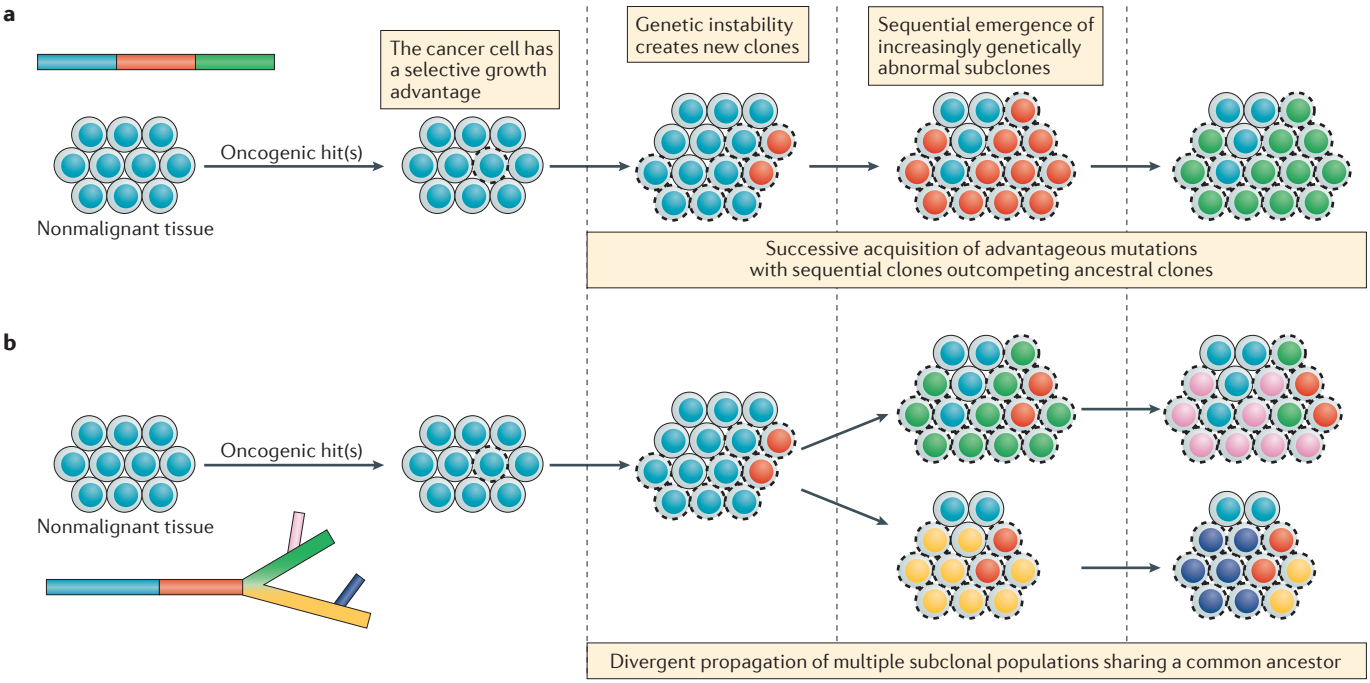

Figure 2 | **Distinguishing between linear and branched tumour evolution.** Two major patterns of evolution exist within the context of the clonal evolution/selection framework. **a** | In the linear evolution model, sequential genetic alterations confer a fitness advantage such that successive generations (red, followed by green) are able to outcompete the preceding clones (blue), which lack this fitness advantage. Surviving dominant clones (green) harbour the ancestral mutations. **b** | In the branched evolution model, multiple genetically distinct populations (green, pink, yellow, purple) can emerge from a common ancestral clone (red), with certain subclonal populations diverging from the common ancestor before others.

outcomes (HR for death 4.9 versus those with ≤48% subclonal copy-number alterations, 95% CI 1.8–13.1; $P = 0.0004$)[6]. Copy-number alterations are a manifestation of underlying genomic instability; therefore, this finding suggests that genomic instability is a better biomarker than the alterations typically detected using gene-expression profiling. However, the in-depth multiregion sampling required to determine the full extent of genomic instability might not be practical or technically feasible for all patients. For example, in one study, the investigators proposed that at least three distinct regions of the same tumour would need to be sampled in surgical resection specimens from patients with clear-cell RCC in order to detect five key mutations with a 90% level of certainty[49]. Most cancers are diagnosed at an advanced stage, when surgical resection is generally not indicated, and attempts to obtain multiple biopsy samples might introduce considerable levels of risk. Therefore, this multiregion sampling approach might be best applied to the investigation of early stage malignancies.

Our current understanding of the extent of intratumoural heterogeneity in cancers is largely derived from analysis of bulk tumour specimens; however, most bulk tumour specimens consist of an admixture of nonmalignant cells and diverse subpopulations of cancer cells. Recognizing the limitations of this type of analysis, several studies have used single-cell sequencing, a novel technology that enables the isolation and characterization of individual cells within a mixed population[50–52]. The high level of resolution provided by this technology has considerable potential for dissecting the multiple dimensions of intratumoural heterogeneity. For example, whole-genome sequencing of single glioblastoma cells has demonstrated the existence of single-cell-level variations in *EGFR* copy number[52]. Furthermore, the single-cell-sequencing approach enabled the authors to deduce that the coexistence of two different oncogenic *EGFR* variants that encode truncated forms of EGFR in the bulk tumour was the result of nonoverlapping subclonal populations of tumour cells harbouring distinct variants. The consequences of heterogeneity at the single-cell level have not been widely studied, although the few published studies that report the use of this technique suggest that a substantial level of genetic diversity exists between individual cancer cells[50,51,53]. Beyond enabling the characterization of cell-to-cell heterogeneity, single-cell sequencing might also enable deconvolution of the complex clonal relationships that are broadly encompassed in the bulk tumour. For example, in a seminal study, Navin and colleagues[50] used single-nucleus sequencing of cells derived from two triple-negative breast cancer specimens to support an evolutionary model whereby clonal subpopulations arise from punctuated clonal expansions (short evolutionary bursts resulting in hundreds of genetic rearrangements) rather than gradual evolution. Quantifying the extent of heterogeneity at the single-cell level could also provide insight into the potential clinical benefits of

genotype-guided therapies. Indeed, the findings of one study demonstrated that the extent of therapeutic benefit derived from T790M-targeting EGFR-tyrosine-kinase inhibitors (TKIs) was proportional to the fraction of cells harbouring the alteration[54]. Despite the advantages of single-cell sequencing over standard approaches to tumour profiling, the current costs, largely stemming from the bioinformatics requirements, might limit the immediate clinical applicability of this method.

Multifocal tumours, or the presence of multiple histologically similar cancers within a single organ, pose a unique challenge because genetic homogeneity cannot be assumed. Moreover, even when a shared genetic signature is apparent, the potential exists for eventual divergence. For example, multiregion sampling of separate foci from four multifocal breast cancers established clonal relatedness, but also revealed the presence of many subclonal, private mutations at high variant allele frequencies in individual foci, suggesting ongoing evolution at distinct disease sites[45]. In some multifocal cancers (such as prostate cancer), individual foci might not share a common clonal ancestry[55]. Morphologically, nonmalignant prostate tissue harbours a high mutational burden; therefore, the presence of multiple clonally unrelated prostate cancers within the same organ might result from clonal expansion of distinct, genetically aberrant populations arising through a field defect[56].

The contribution of pre-malignant populations to the molecular makeup of contiguous malignant cells remains under investigation. Observations from several studies comparing pre-invasive lesions with *bona fide* NSCLCs, oesophageal cancers, and melanomas have demonstrated that the mutational landscape of pre-invasive lesions is distinctly different from that of malignant lesions and that intratumour heterogeneity exists, even within pre-malignant lesions[57–59]. The results of these studies highlight the unique insights into the generation of intratumoural heterogeneity that can be gleaned from comprehensive molecular assessments of multifocal and pre-malignant lesions.

Comparisons of spatially distinct disease sites. Considering the possible extent of heterogeneity within the primary tumour, the genetic makeup of cancer cells at a specific metastatic site might, unsurprisingly, differ from that of the parent tumour or that of other metastatic sites. Indeed, varying degrees of concordance between the genetic makeup of metastatic sites and their primary tumours have been reported across multiple types of solid cancer[37,48,60]. However, owing to the limited number of patients and metastatic sites assessed in these studies, drawing reliable conclusions about the propensity for spatial heterogeneity within a given tumour type or comparing the propensity for spatial heterogeneity across different tumour types is challenging. The degree of genetic discordance between the primary tumour and metastatic sites might reflect whether the metastases occurred as late events or arose through dissemination early in the course of tumour development — the former would be expected to result in a lower level of genetic discordance. In pancreatic cancer, for example, mathematical modelling data suggest that at least a decade is required between the occurrence of an initiating mutation and the appearance of a parental nonmetastatic founder cell and that an additional 5 years are required for the cancer to acquire metastatic potential[37]. Therefore, the distinct subclonal signatures of metastatic lesions can often be traced back to spatially separated founder subclonal populations in the primary pancreatic tumours, with a high level of concordance between the parent tumour and metastatic sites.

In addition to discordance between the primary tumour and metastatic sites, comparisons of the genetic makeup of different metastases often reveal substantial levels of heterogeneity[48,61,62]. In the simplest hypothetical scenario of metastatic outgrowth, which involves seeding of multiple metastatic sites by genetically identical circulating clones, all metastatic sites would have the same genetic signature. For example, a phylogenetic analysis of biopsy specimens from eight patients with ovarian cancer and their associated peritoneal metastases suggested monoclonal seeding from the ovary to the peritoneum in five patients[60]. However, this unidirectional flow from the primary tumour to metastatic sites might not be a universal scenario as the findings of various studies show that tumour self-seeding (recolonization of the primary tumour by circulating tumour cells) and exchange of tumour material between different metastatic sites (referred to as cross-metastatic seeding) can occur[41,43,63]. Furthermore, data from multiple studies have demonstrated the occurrence of polyclonal seeding, or seeding of different metastatic sites by genetically distinct subclones originating from the same primary tumour[43,64]. Indeed, the assumption that clones located at distant metastatic sites derive from prior colonization of locoregional nodes has been challenged by the observation, from a small series of patients, that two-thirds of distant CRC metastases are genetically distinct from metastases located in tumour-draining lymph nodes[64]. These findings suggest that, in some cases, distant metastases and lymph-node metastases arise from independent seeding by genetically distinct subclones originating from the primary tumour.

Moreover, even when cancer cells at locoregional sites and distant sites share a common ancestor, site-specific factors (such as the interaction between cancer cells and other cells in the local microenvironment) could promote genetic divergence after initial colonization of metastatic sites. For example, in a large series comparing the genetic characteristics of primary tumours, brain metastases, and extracranial metastases, the authors observed branched evolution of brain metastases resulting in genetic uniformity among different brain metastases despite considerable genetic divergence from extracranial metastases[65]. Notably, extensive sampling of extracranial metastases, in order to comprehensively assess genomic relationships, was not performed. Nevertheless, the findings of this study and of others demonstrate that cancer cells in the same tissue are more closely related than those in different

tissues, and suggest a potential role of tissue-specific factors or proximity in influencing the evolutionary trajectory of disseminated cancer cells[31,43].

Insights from autopsy sample analysis. Biopsy sampling of multiple regions of the same tumour and of multiple metastatic sites has not been widely adopted owing to the prohibitive risks associated with the acquisition of multiple biopsy samples. Molecular analysis of biopsy samples from multiple tumour sites acquired during autopsy offers an alternative strategy for the comprehensive assessment of spatial heterogeneity. Indeed, the handful of autopsy series published to date has substantially increased our appreciation of the marked degree of genetic heterogeneity that can exist across metastatic sites[7,66–69]. To date, molecular analysis in these studies has largely focused on uncovering mechanisms of resistance to targeted therapies. These cases represent a biologically distinct subset of cancers and might not reflect the heterogeneity that arises independent of the presence of potent selective pressure. In one series, sampling of metastatic sites from a patient with breast cancer harbouring an activating *PIK3CA* mutation who developed progressive disease in multiple sites while receiving a PI3Kα inhibitor revealed the emergence of multiple distinct *PTEN* mutations at metastatic sites through parallel evolution[7]. In this patient, the isolated clonal ecosystem of individual metastatic sites enabled the coexistence of site-specific clones that conferred the same evolutionary advantage.

In addition to elucidating the capacity for heterogeneity, data from autopsy studies suggest that the degree of heterogeneity varies widely and is affected by the patient, their treatment, and the characteristics of the underlying disease. For example, sequencing of multiple metastatic sites in a patient with NSCLC harbouring a *CD74–ROS1* rearrangement who developed resistance to crizotinib, a multitargeted inhibitor of MET, ALK, and ROS1, revealed the same *ROS1*^{G2032R} mutation at all sampled metastatic sites[70]. This finding might have resulted from either seeding of all metastatic sites by genetically identical circulating clones or parallel evolution at distinct sites; although, in this patient, the former scenario seems most likely becuase the same genetic profile (including the *ROS1*^{G2032R} mutation) was identified at every site via targeted sequencing using a 409-gene panel. Notably, other autopsy studies have yet to demonstrate this striking degree of genetic homogeneity. However, the number of patients included in published autopsy series to date has been limited. Observations from one individual might not be broadly applicable to all patients, as host-specific factors might modulate the effects of selective pressures; therefore, it will be important to generate autopsy analyses from multiple patients with cancers exposed to the same stressors. Notably, in the aforementioned breast cancer autopsy series, intertumoural heterogeneity of resistance mechanisms was observed, with some patients developing resistant lesions harbouring clones lacking the original *PIK3CA* mutation, while others developed *PTEN* mutations[7].

Temporal heterogeneity

Insights on the effects of temporal heterogeneity, a term that refers to the dynamic variation in the genetic diversity of a tumour over time (FIG. 1b), provided the groundwork for the analyses of spatial heterogeneity described above. Data from studies employing serial biopsy sampling to characterize the evolution of tumours have demonstrated that chemotherapy can alter the molecular makeup of tumours over time by creating shifts in the mutational spectrum[17–19]. In particular, mutations in genes that are fundamental to replication and cell-cycle regulation can contribute to genomic instability. For example, treating glioblastomas with temozolomide can enrich for transition mutations in MMR genes, leading to the development of a hypermutated phenotype[17]. Treatment with targeted therapies might exert more-potent selective pressures on oncogene-driven cancer cells than nonspecific therapies such as cytotoxic chemotherapy. Indeed, many of the most compelling observations regarding temporal heterogeneity have occurred within the context of treatment with targeted agents.

Genomic complexity might increase with exposure to targeted therapies. The incorporation of molecularly targeted therapies into clinical practice has improved the prognosis of certain subgroups of patients with solid tumours, many of which were previously associated with dismal outcomes[4,71]. The remarkable efficacy of these targeted therapies largely reflects therapeutic vulnerabilities resulting from a dependence on specific growth signals and the truncal location of the driver alteration (such as *EGFR* mutations in NSCLCs), specifically the presence of such mutations in the founder clone. Despite dramatic responses to initial therapy, however, relapse typically occurs within 1–2 years of starting treatment. Resistance can arise through many different mechanisms, including acquired mutations, activation of bypass signalling pathways, and cell-lineage changes[8,72–74]. Although these resistance alterations are often thought of as 'acquired', the findings of several studies have identified *de novo* resistance alterations that are present at low variant allele frequencies in pretreatment tumour specimens[75,76] (FIG. 3a). In addition, the findings of preclinical cellular barcoding experiments confirm that resistant clones often emerge from the selective expansion of pre-existing populations during treatment with targeted agents[5]. The genomic complexity of a tumour generally increases with exposure to sequential systemic therapies; therefore, the single genetic snapshot depicted in a diagnostic biopsy sample might become outdated during the clinical course[17–19]. Thus, serial characterization of tumours at multiple timepoints is necessary in order to accurately capture the various temporal shifts that take place during clonal evolution.

Longitudinal sampling provides insight into temporal heterogeneity. Longitudinal profiling of tumours has tremendous potential to decipher the role of clonal evolution in the development of treatment resistance and to untangle the complex interplay between treatment selection and clonal shifts. Clonal evolution

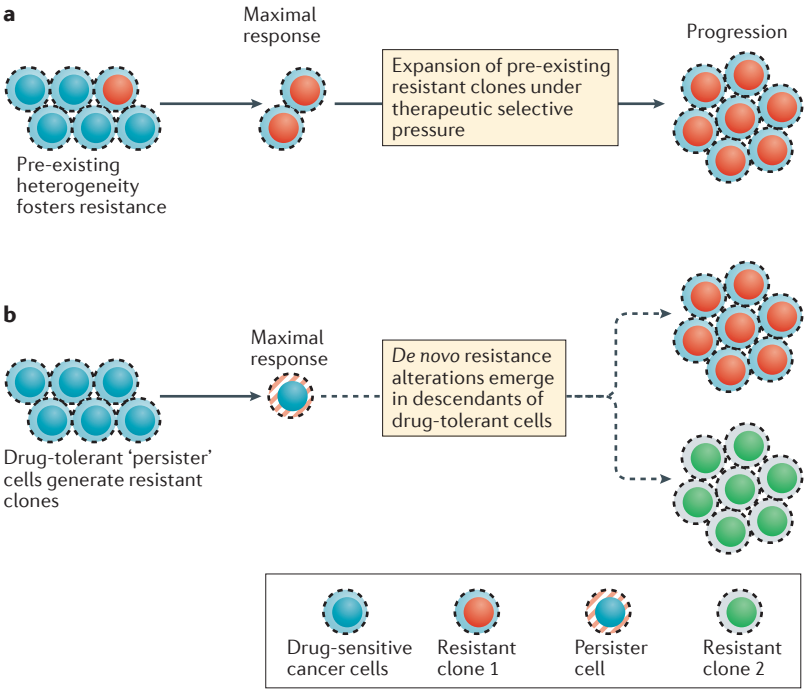

Figure 3 | **Resistance arises from two distinct evolutionary pathways. a** | Outgrowth of pre-existing treatment-resistant clones (red) during treatment. **b** | Alternatively, drug-tolerant 'persister' cells (blue with orange stripes) that survive initial treatment acquire additional alterations (red or green) that confer resistance to therapy.

that arises from the selective pressures created by targeted agents is dynamic, with the emergence, loss, and reappearance of clones governed by the specific choice and administration schedule of therapeutic agents[73,77,78]. For example, Sequist and colleagues[73] described a patient who developed an *EGFR*^{T790M} resistance mutation during treatment with the EGFR TKI erlotinib; however, this mutation was no longer detected on analysis of a repeat biopsy sample obtained after a 10-month drug-free interval[73]. This patient then briefly responded to rechallenge with erlotinib, although *EGFR*^{T790M} again became detectable at the onset of resistance to erlotinib[73]. The reversal of resistance after dose interruption probably reflects clonal shifts in the absence of the selective pressure created by continuous treatment with erlotinib. Rechallenge does not typically produce durable responses, but this and other cases demonstrate how serial molecular characterization might enable the implementation of rational therapeutic approaches. Subsequent studies investigating resistance to third-generation EGFR TKIs have demonstrated similar selective pressures on *EGFR*^{T790M}-mutant cancer cells, suggesting that tumours harbouring this alteration are particularly prone to clonal plasticity[54,79].

Clonal dynamics are not always easily manipulated by treatment interruption; therefore, longitudinal sampling might be most clinically relevant when used as a tool to enable the selection of subsequent treatment strategies. In patients with *ALK*-rearranged NSCLC, for example, a much larger spectrum of *ALK* resistance mutations is observed among patients who develop resistance to targeted agents[72]. The resistance mutation profile is unique for each ALK TKI, and repeat biopsy sampling enables the tailored use of sequential therapies. The sequential use of targeted therapies can be quite beneficial, although the successive use of multiple TKIs can lead to the emergence of highly resistant compound mutations. Interestingly, a patient with *ALK*-rearranged NSCLC developed a compound resistance mutation after consecutive treatment with three different ALK TKIs, including crizotinib, which conferred high-level resistance to all next-generation ALK TKIs but resensitized the cancer to crizotinib[75]. This event has only been reported once. However, this case report and the body of experience with repeat biopsy sampling in oncogene-driven cancers underscores the clinical utility of repeat biopsy sampling for capturing the full extent of temporal heterogeneity and identifying the most appropriate therapeutic options.

Residual drug-tolerant cells can foster temporal heterogeneity. To date, assessments of temporal heterogeneity have primarily relied on analysis of biopsy samples obtained from the sites of treatment-resistant disease. Such an approach requires lesions large enough to obtain biopsy samples; hence, a reliance on this strategy alone might fail to detect cancers at the early or intermediate stages of resistance. Even when the most-effective therapies are used, most patients have an incomplete tumour response and are left with a residual tumour mass. This residual disease could harbour a small population of quiescent drug-tolerant cells that have survived owing to adaptive activation of alternative metabolic pathways, survival signals, and epigenetic programmes[80–86]. Acquired resistance to antineoplastic treatments is often attributed to selective expansion of pre-existing subclonal populations; however, data from two preclinical studies suggest that the ongoing evolution of drug-tolerant cells leads to *de novo* generation of resistance alterations[87,88] (FIG. 3). In these studies, the authors showed that alterations typically associated with resistance to EGFR TKIs, including *EGFR*^{T790M} and *MET* amplification, can selectively emerge from single-cell clones derived from drug-tolerant cells. This finding emphasizes the necessity of developing sensitive technologies that enable the early detection of resistance. Additionally, the emergence of resistance from drug-tolerant cells highlights the need to develop therapeutic strategies that target the minimal residual disease state.

Noninvasive monitoring of heterogeneity

The clinical status of a patient and the location of progressing lesions might make performing a repeat tissue biopsy procedure infeasible. Moreover, analyses involving single-site biopsy sampling might result in underestimation of the degree of spatial heterogeneity, and sampling intervals that are tolerated by the patient might not enable the true extent of temporal heterogeneity to be captured accurately. Noninvasive 'liquid biopsies' that facilitate longitudinal analysis of tumour-derived genetic material extracted from a patient's blood are a promising strategy for addressing the shortcomings of tissue sampling. Genotyping of genetic material obtained from

circulating tumour cells, circulating exosomes shed by tumours, and circulating cell-free tumour DNA (ctDNA) has yielded promising results across several types of solid tumours[89–93]. However, much of the research exploring the potential of liquid biopsies for improving understanding of intratumoural heterogeneity has involved ctDNA; therefore, our discussion will focus on analysis of ctDNA.

Analysis of ctDNA

Data from multiple studies have established analysis of ctDNA as a sensitive and highly informative method of identifying clinically relevant genomic alterations with a high degree of concordance with the findings of tissue biopsy sample analysis in patients with metastatic cancer[94–96]. In some scenarios, ctDNA might enable the identification of alterations that were not detected by tissue genotyping, some of which have therapeutic implications. For example, in a prospective study involving 121 patients with metastatic CRC, 26 patients were found to have *KRAS* mutations in plasma that were not identified by tissue genotyping[97]. Optimizing ctDNA platforms to increase sensitivity for very-low-frequency mutations might enable the early detection of resistance and relapse in greater numbers of patients[98–100]. Indeed, the detection of variants associated with treatment resistance in plasma can precede the emergence of evidence of radiographic progression by 10 months in some patients with metastatic CRC[101,102]. The sensitivity of ctDNA platforms had previously limited the application of ctDNA-based analyses in patients with localized cancers, although developments in the past year have improved the yield in patients with early stage cancers. For example, ctDNA was detected preoperatively in 46 (48%) of 96 patients with early stage NSCLC using personalized multiplex PCR panels[100]. Notably, in addition to detecting preoperative plasma variants, the authors detected evidence of postoperative relapse in plasma samples at an average of 70 days before radiographic recurrence, with some cases preceding imaging-confirmed recurrence by >6 months[100]. These findings are encouraging; although, further refinements are necessary before ctDNA can be broadly applied as an early detection strategy. In support of the challenges of using this technology to detect disease progression and the current limitations of applying this technology to cancer screening, linear modelling based on early stage NSCLC predicted that a tumour burden of ≥10 cm^3, or approximately 326 million cells, is necessary to detect alterations present at a 0.1% variant allele frequency, a value that is at the lower limit of detection for most assays[100].

Longitudinal plasma analysis is an effective tool for gauging the influence of treatment on the molecular and/or genetic makeup of a patient's cancer over time. For example, several reports suggest that the plasma $EGFR^{T790M}$ allelic fraction decreases over time in patients treated with $EGFR^{T790M}$-specific inhibitors, such as the third-generation EGFR inhibitor osimertinib, an observation that might reflect the negative selection of $EGFR^{T790M}$-positive subclones[79,90]. In fact, plasma clearance might be predictive of a clinical response. In support of this suggestion, clearance of $EGFR^{T790M}$ after 6 weeks of treatment is associated with a statistically significant increase in overall response rate (70% versus 35%, 95% CI 59–79% versus 22–50%; $P < 0.05$) and median progression-free survival (PFS) duration (10.9 months versus 5.5 months, 95% CI 9.5–15.2 months versus 3.9–6.7 months; $P < 0.05$) on osimertinib[103]. Similarly, in another study, the persistence of detectable alterations in ctDNA during adjuvant chemotherapy predicted the occurrence of disease relapse within 1 year of surgery[100]. In addition to enabling the kinetics of dominant alterations that were present before treatment to be monitored, serial plasma analysis has the capacity to capture clonal shifts occurring during therapy. For example, treatment of a patient with *MET*-amplified oesophageal cancer with a MET inhibitor resulted in suppression of *MET* copy number and a marked increase in plasma *EGFR* copy number[8]. As predicted by plasma monitoring, analysis of ascites that developed at progression revealed the presence of clones harbouring *EGFR* amplification but lacking *MET* amplification. In another study, serial profiling of plasma samples from patients with CRC treated with anti-EGFR monoclonal antibodies demonstrated that *KRAS* mutations emerged when tumours became resistant to treatment and disappeared when EGFR blockade was withdrawn[104]. Notably, many of these *KRAS* mutations are thought to predate treatment and are predictive of a poor response to anti-EGFR monoclonal antibodies[105]. The findings of the studies described above, and those of others, confirm that genotyping of plasma samples enables the kinetics of intratumoural heterogeneity to be captured in a timeframe that is potentially conducive to guiding clinical decision-making.

Plasma samples, theoretically, contain ctDNA from multiple metastatic sites; therefore, genotyping of plasma samples can enable the detection of clinically relevant alterations that are not identified through analysis of tissue biopsy samples[69] (FIG. 4). In one study mentioned above, none of the 26 patients with discrepant *KRAS* status (in which a *KRAS* mutation was detected in plasma but not identified in tissue) responded to treatment with an anti-*EGFR* monoclonal antibody, and several had progressive disease as a best response[97]. Determining *KRAS* mutation status in patients who have progressed on EGFR-directed therapy is equally important. Here, again, analysis of plasma samples might offer an advantage. Indeed, new *KRAS* mutations were observed in 7.1% versus 57.1% of patients whose tumour genotype was determined using tissue-based and plasma-based analyses, respectively, during treatment with the combination of irinotecan and panitumumab[106]. Similarly, the $EGFR^{T790M}$ mutation is routinely identified in plasma samples from patients with concurrent biopsy samples that are negative for this alteration[89,93]. Patients with $EGFR^{T790M}$ detected in plasma have almost identical outcomes to those of patients with the same mutation detected in tissue samples, confirming that the presence of $EGFR^{T790M}$ in plasma is a valid biomarker[89]. Interestingly, approximately 50% of patients with $EGFR^{T790M}$ might have additional alterations that are detectable in plasma samples that confer

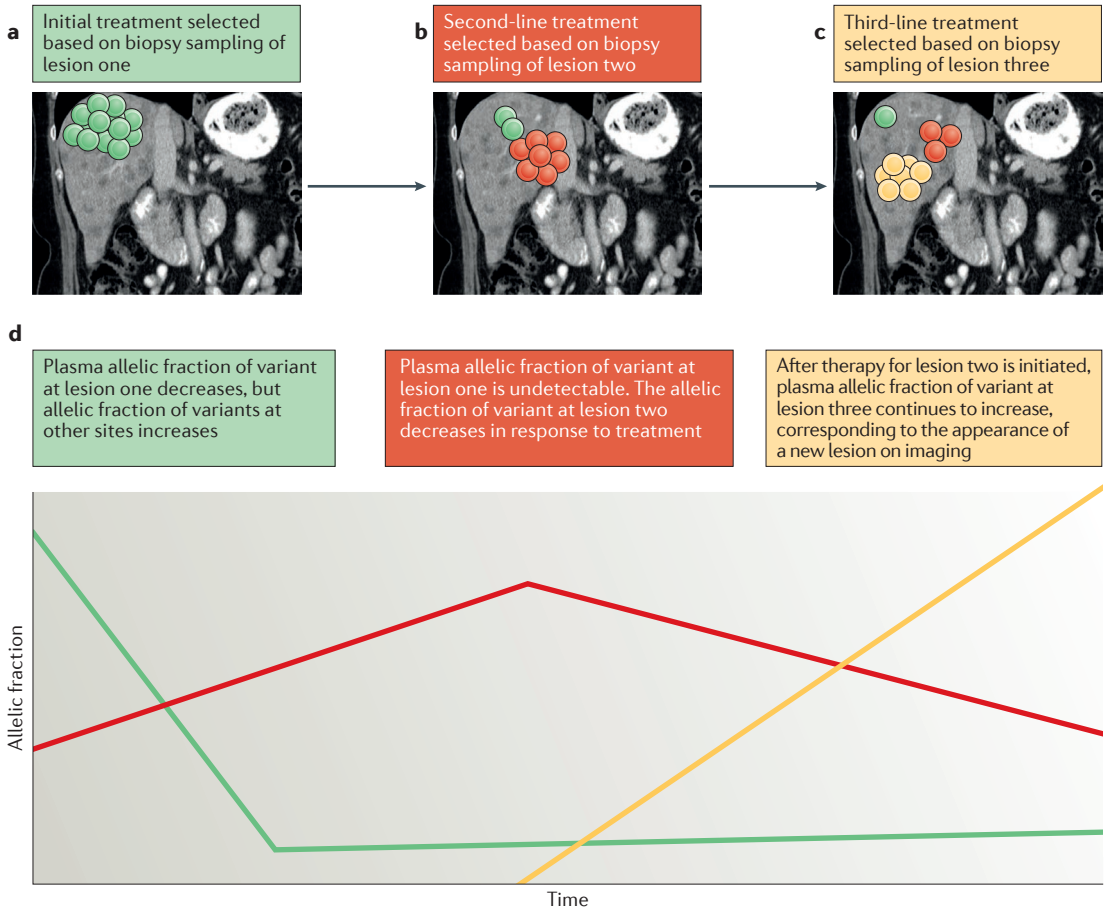

Figure 4 | **Application of longitudinal plasma profiling.** Longitudinal monitoring of alterations in circulating tumour DNA has the potential to enable molecular relapses to be detected before the emergence of disease relapse on imaging. In a hypothetical example: **a** | A biopsy sample from lesion one (green) leads to the use of a targeted agent directed at the alterations in lesion one. **b** | A failure to also sample lesion two (red) might then lead to outgrowth of clones harbouring alternative molecular alterations, prompting the use of a combination of targeted agents or use of a single targeted therapy capable of overcoming both molecular alterations. **c** | The emergence of lesion three (yellow) might then be missed by biopsy sampling until this lesion becomes detectable on imaging. **d** | Longitudinal analysis of liquid biopsy samples would enable the detection and determination of the allelic fractions of the variants at all three lesions before their detection on imaging. This figure illustrates the ability of the molecular analysis of plasma to convey the full spectrum of resistance alterations and shows the dynamic nature of resistance.

resistance to EGFR inhibition, including *EGFR, MET*, and/or *HER2* amplification, that might not be detected in tissue biopsy samples[90]. This observation supports the notion that reliance on tissue sampling alone often underestimates the degree of overlap between distinct driver alterations. Similar to tissue biopsy studies, heterogeneity of alterations associated with resistance in plasma samples (such as the presence of multiple molecular alterations conferring resistance to EGFR inhibition) correlates with shorter PFS durations among patients receiving $EGFR^{T790M}$-specific inhibitors[90]. This finding suggests that analysis of pretreatment plasma samples using next-generation sequencing can provide some insight into the probable disease outcomes of patients receiving treatment with $EGFR^{T790M}$-specific inhibitors and potentially identify patients that require close monitoring.

Sampling of multiple lesions during autopsy has the potential to improve upon the ability of tissue sampling to capture the extent of molecular heterogeneity present in cancers. Nonetheless, plasma genotyping might provide a more-comprehensive readout of tumour heterogeneity than autopsy-based analyses[67]. In one study involving patients with *FGFR*-amplified cholangiocarcinoma, five unique *FGFR2* mutations were identified in plasma samples obtained at the onset of resistance to infigratinib, an FGFR inhibitor, whereas sampling of 12 distinct sites during autopsy revealed only three *FGFR2* mutations[67]. In summary, the sensitivity of the technology, the accessibility of biopsy material relative to multisite sampling, and the ability to accurately convey spatial and temporal heterogeneity make longitudinal analysis of ctDNA an appealing alternative to serial tissue biopsy sampling.

Overcoming heterogeneity

The findings of multiple studies demonstrate that higher levels of intratumoural heterogeneity predispose patients to inferior responses to anticancer therapies, including

to targeted agents[47,54,89,90,107,108]. For example, when the baseline fraction of *EGFR*^{T790M}-positive cells was calculated based on the ratio of the allelic frequencies of the *EGFR*^{T790M} 'gatekeeper' resistance mutation to the activating *EGFR* mutation, a higher baseline fraction of *EGFR*^{T790M}-positive cells correlated with greater tumour shrinkage on treatment with a third-generation EGFR inhibitor[54]. *EGFR*^{T790M}-positive and *EGFR*^{T790M}-negative cells can co-occur, and this finding suggests that the degree to which these populations coexist will affect clinical outcomes (FIG. 5). Cancers generally become more heterogeneous and genomically complex with successive exposure to systemic agents. As a consequence, responses to subsequent lines of therapy are often not as robust as responses to initial treatment. Even among oncogene-addicted cancers, heterogeneity can develop and can undermine the therapeutic efficacy of potent and selective TKIs. This widespread observation suggests that the current paradigm of sequential treatment with TKIs could be suboptimal, as it fails to address the heterogeneity that might underlie incomplete responses to treatment. Instead, upfront treatment with potent pan-inhibitory TKIs might be more efficacious than reserving these agents for the second-line and beyond. In a phase III study, upfront treatment with the second-generation ALK TKI alectinib improved response rates from 76% to 83% and more than doubled the median PFS duration of a group of patients with ALK-positive NSCLC[109]. Similarly, first-line treatment with osimertinib resulted in a median PFS duration of 19.3 months in a pooled analysis of data from two phase I expansion cohorts, a value that is approximately twice as long as that expected with use of first-generation or second-generation EGFR TKIs[110]. This strategy might be highly effective for delaying the onset of resistance, although it is unlikely to completely extinguish resistant clones. Rather, treatment with more-potent TKIs might simply divert the course of clonal evolution. For example, the findings of one preclinical study demonstrate that *EGFR*^{T790M}-negative cells with an acquired *EGFR*^{C797S} mutation emerged during upfront treatment with a third-generation EGFR TKI[111].

A bulk solid tumour is a heterogeneous entity that predominantly consists of drug-sensitive cells; therefore, mathematical modelling might enable the design of dosing schedules that account for this inherent heterogeneity. Central to these models is the concept that withdrawal of targeted therapy can negate the selective advantage conferred upon drug-resistant cells, and enable repopulation of the tumour with drug-sensitive cells. In a patient-derived xenograft model of melanoma, a complex dosing schedule consisting of intermittent doses of a BRAF inhibitor delayed the onset of resistance and led to prolonged disease stabilization[112]. As a proof-of-principle, intermittent dosing with the BRAF and MEK inhibitors vemurafenib and cobimetinib in a patient with concurrent *BRAF*-mutant melanoma and *NRAS*-driven leukaemia suppressed the proliferation of leukaemic cells and led to a near-complete remission of the melanoma[113]. Similarly, dose interruption can restore sensitivity to EGFR TKIs[73,114]. The use of intermittent dose scheduling can temporarily suppress clonal outgrowth, although the overall effect is to subdue heterogeneity rather than to eliminate it altogether. These observations suggest that combination strategies that target the small pre-existing population of drug-resistant cells, in addition to the predominantly drug-sensitive population, are likely to be most effective.

Combination approaches that target heterogeneous tumour populations have proven successful in preclinical studies. For example, upfront treatment with the combination of the second-generation EGFR TKI afatinib and the anti-EGFR monoclonal antibody cetuximab, a drug combination with preclinical activity against tumour cells harbouring both sensitizing *EGFR* mutations and the *EGFR*^{T790M} resistance mutation, lengthened the time to relapse and delayed the emergence of the *EGFR*^{T790M} mutation in a mouse model of *EGFR*-mutant lung adenocarcinoma[115]. Notably, this combination has modest activity and results in considerable levels of toxic effects when administered to patients who have developed resistance to first-generation EGFR TKIs[116]. In the case of chronic myelogenous leukaemia, combinations involving an ABL1 TKI and an allosteric inhibitor of ABL1 that does not target the ATP-binding site and has a nonoverlapping resistance profile suppressed

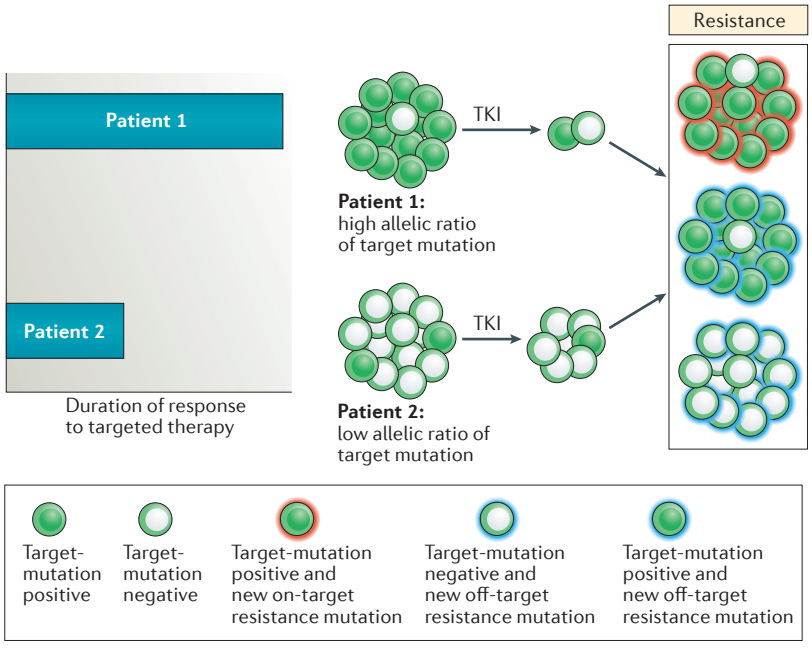

Figure 5 | **Correlation between pretreatment tumour heterogeneity and response to targeted therapies.** When the proportion of cells bearing the target alterations is compared with the proportion of cells with the wild type form of the altered allele (the allelic ratio), a higher allelic ratio (patient 1) is associated with a more durable response to treatment than a lower allelic ratio (patient 2). Regardless of the baseline distribution of cells bearing the target mutation and that of cells negative for the target mutation, resistance might result from the outgrowth of target-mutation-positive cells, outgrowth of target-mutation-negative cells, or the acquisition of a secondary on-target resistance mutation. Acquired off-target alterations could drive the development of resistance in patients that do not acquire a secondary on-target resistance mutation. TKI, tyrosine kinase inhibitor.

the outgrowth of mutations associated with resistance and resulted in sustained responses in mouse models[117]. If effective in the clinical setting, such combinations might address the issue of pre-existing heterogeneity and thwart the development of resistance secondary to on-target resistance mutations.

Plasticity between different signalling pathways, specifically the adaptive activation of bypass signalling, is a potential manifestation of temporal heterogeneity under therapeutic selective pressure[8,73,74,90]. As such, drug combinations targeting multiple signalling pathways could provide another means of addressing intratumoural heterogeneity. In support of this suggestion, two groups independently reported the delayed emergence of resistance in cellular models of *EGFR*-mutant or *ALK*-positive NSCLC exposed to the combination of a next-generation TKI and a MAPK inhibitor[118,119]. In *EGFR*-mutant NSCLC, cells harbouring *MET* amplifications might be present before treatment or can arise from drug-tolerant cells[87,120]. The ability of an $EGFR^{T790M}$-specific inhibitor in combination with a MET TKI to suppress the outgrowth of resistant cells bearing alterations in either protein is currently being explored in an ongoing clinical trial (NCT02335944). The findings of one study suggest the existence of variations in the growth kinetics of TKI-sensitive and TKI-resistant tumour-cell populations[114]. These observations provide the rationale for exploring intermittent or intercalated dosing schedules, particularly as these schedules may improve the tolerability of TKI monotherapy or TKI-based combination regimens, relative to continuous dosing.

In addition to targeting pathways and alterations commonly implicated in resistance, the characteristics of the tumour before treatment could, theoretically, be used to design therapeutic approaches. For example, based on data from several studies, the ratio of $EGFR^{T790M}$ to the activating *EGFR* mutation is correlated with outcomes[54,90]; this ratio could potentially be developed into a biomarker that enables the selection of patients to receive osimertinib monotherapy. Patients with tumours that have a high allelic ratio might derive the greatest level of benefit from treatment with osimertinib monotherapy whereas those with tumours that have a low allelic ratio could potentially derive greater levels of benefit from inclusion in investigational trials of combination treatments because the use of osimertinib monotherapy in this subgroup could, theoretically, favour the outgrowth of $EGFR^{T790M}$-wild-type cells that rely on bypass signalling. Interestingly, the findings of one study revealed a correlation between inactivating *TP53* and *RB1* mutations and transformation from *EGFR*-mutant NSCLC to small-cell lung cancer (SCLC)[121]. A pre-existing small-cell population has not been identified in patients with NSCLC harbouring such mutations, although the authors found that loss of *TP53* and *RB1* expression could both be detected well before transformation to SCLC[121]. Establishing whether regimens containing platinum plus etoposide, as opposed to the more traditional platinum plus pemetrexed combination can prevent histological transformation in patients with high-risk NSCLC might be informative. However, chemotherapy is generally reserved for patients in whom EGFR-directed therapies have been exhausted, and it currently remains unclear as to whether chemotherapy alone would impose the same selective pressures that fuel this histological transformation.

Drug-tolerant cells can develop a wide range of resistance mechanisms and, therefore, targeting this population offers another opportunity to curtail intratumour heterogeneity. Combinations are likely to be more effective than monotherapy against drug-tolerant cells. For example, Hata and colleagues[88] demonstrated that while drug-tolerant cells that acquired $EGFR^{T790M}$ have a decreased level of sensitivity to $EGFR^{T790M}$-specific inhibitors, the combination of an $EGFR^{T790M}$-specific inhibitor with navitoclax, a drug targeting apoptosis, restored this sensitivity. A clinical trial exploring the efficacy of osimertinib plus navitoclax is currently underway (NCT02520778). In a different study using similar models of *EGFR*-mutant NSCLC, the authors proposed that co-activation of signal transducer and activator of transcription 3 (STAT3) and SRC signalling promotes the development of a residual disease state and showed that co-targeting of EGFR, STAT3, and SRC has greater antitumour effects than EGFR monotherapy[122]. In preclinical models of *BRAF*-mutant CRC, treatment with a MEK inhibitor led to an adaptive increase in MET–STAT3 signalling, resulting in upregulation of prosurvival signals that were overcome by the combination of a MEK inhibitor and the HDAC inhibitor vorinostat[123]. In addition to exploring the efficacy of novel combinations, the use of locally ablative therapies has also been considered for the eradication of residual disease. Randomized studies exploring this approach are limited, although the findings of one study revealed an improvement in median PFS duration from 3.9 months to 11.9 months in selected patients with NSCLC with a limited disease burden who received consolidative radiation therapy delivered to sites of residual disease[124].

Many tumours lack actionable genetic alterations. In these cases, strategies that target more ubiquitous sources of heterogeneity are likely to be most applicable. Genomic instability is a pervasive and ideal target; however, the multitude of genes involved in maintaining genomic integrity and the inevitability of exposure to extrinsic sources of potential heterogeneity during a patient's clinical course suggest that countering the development of genomic instability is a more daunting task than silencing a dominant signalling pathway. Targeting genomic instability is likely to be most effective in patients with cancers that are particularly prone to mutagenic stress, such as those with deficient DNA repair mechanisms. For example, data from preclinical studies suggest that treatment with inhibitors of CHEK1, a DNA-replication stress protein, is a rational approach for patients with cancers susceptible to APOBEC3-induced mutagenesis[26]. Moreover, the high somatic mutation burden created by genetic instability could be ideally suited to immune-checkpoint inhibition. Indeed, the findings of several clinical studies and case reports have revealed

excellent responses to immune-checkpoint blockade among patients with MMR-deficient cancers and hypermutated tumours resulting from *POLE* deficiency[125–127]. On the basis of promising findings from several clinical trials, including a 40% overall response rate in patients with MMR-deficient CRC (compared with 0% in patients with MMR-proficient CRC) following treatment with pembrolizumab[126], nivolumab and pembrolizumab have both received FDA approval for the treatment of patients with MSI-high and/or MMR-deficient CRCs. Notably, the approval of pembrolizumab allows the use of this agent in patients with MSI-high and/or MMR-deficient tumours, irrespective of histology[128].

Conclusions

Malignant transformation requires the integrated destabilization of several key cellular regulatory processes. The resulting genetic instability creates the necessary substrate for heterogeneity, which is subsequently maintained by selective processes, including therapeutic selective pressures. This heterogeneity underlies resistance and site-specific responses and also complicates the selection of globally effective therapeutic agents. Even in the simplest scenario of an oncogene-driven cancer, heterogeneity ultimately provides the seeds for the emergence of resistance and, eventually, disease relapse. The manifestations of resistance are as diverse as the underlying cancers and range from a single, identical, resistant subclone that is present at all metastatic sites to an assortment of distinct subclones that are present at different anatomical sites. Even when alterations conferring treatment resistance are identified and agents that target these vulnerabilities are selected, further evolution induced by treatment-related selective pressures enables escape from growth suppression. Indeed, regardless of the mechanism of administered therapies (either targeted agents, chemotherapy, or immunotherapy), resistance is a near-universal occurrence in patients with cancer.

These observations support the notion that therapeutic approaches must be as multidimensional as the cancers that they are designed to overcome. Clinical practice must also be dynamic, with timely adjustments of antineoplastic strategies becoming the new norm. The fuel for relapse can be present at the earliest stages of tumorigenesis, and diversity increases throughout the natural history of a cancer; therefore, the timing of a specific intervention is critical. Upfront combination strategies co-targeting the dominant drug-sensitive population and other pre-existing resistant subclones have shown early promise in preclinical studies, although drug-tolerant cells might ultimately still develop and give rise to resistance. As such, the next generation of cancer treatments should aim to eradicate heterogeneity and address the residual disease state. On the basis of our current understanding of the evolution of resistance and the underpinnings of the drug-tolerant state, combination therapies, including combinations of systemic and local therapies, might hold the greatest level of promise.

Inferences of clonal dynamics from autopsy specimens and tissue samples obtained from sites of resistance have tremendous potential to guide the development of treatment strategies that address tumour heterogeneity. The barriers to performing tissue biopsy at regular intervals and to sampling multiple sites suggest, however, that this approach should ideally be combined with use of noninvasive liquid biopsy sampling, which enables more-frequent surveillance. The practical utility of liquid-biopsy-based analyses is as much dependent on the ability to improve surveillance as it is on the ability to facilitate interventions that improve patient outcomes. The prognostic implications of isolated relapse detected in plasma samples remain largely unknown; therefore, trials designed to investigate the benefits of early interventions targeting subclinical relapse are needed. Additional refinement is necessary to improve upon the current level of sensitivity of liquid biopsy assays. Still, this technology might eventually be an ideal platform for determining the full extent of tumour heterogeneity, tracking the emergence of treatment-resistant subclones, and selecting therapies that can prevent and/or overcome resistance. Finally, given the complexity of cancer and the seemingly endless arc of evolution, it is equally important to integrate the lessons learned from patients into models, both preclinical and computational, that enable us to better predict the evolutionary trajectory of a cancer and allow us to design more effective and durable anticancer therapies.

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