Deciphering Genetic Intratumor Heterogeneity and Its Impact on Cancer Evolution

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Abstract

Cancer is a disease reliant on the generation of mutations and the subsequent selection of those subpopulations endowed with the greatest fitness advantage. Beginning with a heterogeneous landscape of somatic alterations, various selective pressures acting on a tumor can shape the way it evolves. In this review, we first discuss the current bioinformatics tools available to tease apart the heterogeneous nature of a tumor and second consider the impact that evolutionary forces have on sculpting a tumor. Neighboring subclones may alter the microenvironment cultivating either cooperation or competition between clonal populations. Additionally, the harsh environment brought about by therapy and the immune system may force adaptation. Finally, we examine recent analyses focused on precancerous samples, which help to reveal clonal selection occurring during the earliest stages of tumor development, as well as work that has identified patterns of somatic evolution observed in normal tissues.

INTRODUCTION

Tumor development is an evolutionary process, involving the twin forces of mutation generation and selection. One hallmark of this Darwinian process is tumor heterogeneity, which provides the fuel upon which selection acts. Although mutation generation necessarily leads to heterogeneity, microenvironmental, immune, and therapeutic selection pressures can dynamically sculpt a tumor as it develops, fostering either homogeneity or heterogeneity. Recent studies have begun to shed light on the extent of diversity within solid and hematological tumors, revealing that many tumors develop through a process of branched clonal evolution, in which distinct clonal populations coexist, possibly competing or cooperating during a cancer's progression. The wealth of data from next-generation sequencing studies has also shed light on the processes involved in generating mutations (Alexandrov et al. 2013, Helleday et al. 2014, Lawrence et al. 2013, Segovia et al. 2015) and the dynamics of tumor clones during the disease course and through treatment (Calbo et al. 2011, Keats et al. 2012, Landau et al. 2013, Marusyk & Polyak 2010, Murtaza et al. 2013).

In this review, we focus on the role of selection and clonal evolution in generating, maintaining, or eliminating tumor heterogeneity. We explore tumor evolution, intratumor heterogeneity (ITH), and clonal dynamics in the context of precancer, immune control, and through therapy. We also investigate the tools to analyze heterogeneous tumor genomes and the insights these tools have provided to the field.

Although macroscopic ITH has likely been evident since tumors were first excised many thousands of years ago (Mukherjee 2011), an understanding of the extent of diversity at the single-nucleotide level within tumors has only fully emerged as a result of next-generation sequencing. Recent in-depth studies of single tumor samples, as well as multiple and serial sampling techniques, have revealed considerable variability in the extent of diversity within both patients and individual tumors. Next-generation sequencing has allowed for the identification and characterization of genetic ITH across a wide range of cancer types, including breast carcinomas (Navin et al. 2011, Nik-Zainal et al. 2012b), clear-cell renal carcinomas (Gerlinger et al. 2012, 2014a), glioblastomas (Sottoriva et al. 2013), gliomas (Johnson et al. 2014), prostate cancers (Gundem et al. 2015, Haffner et al. 2013), non-small-cell lung cancers (NSCLCs; de Bruin et al. 2014, Zhang et al. 2014), head and neck squamous cell carcinomas (Mroz et al. 2015), squamous cell melanomas (Ding et al. 2014), high-grade serous ovarian cancer (Schwarz et al. 2015), chronic lymphocytic leukemia (CLL; Landau et al. 2013), acute myeloid leukemia (Ding et al. 2012, Klco et al. 2014), and multiple myeloma (Bolli et al. 2014, Lohr et al. 2014). Taken together, these studies have demonstrated that heterogeneity is observed to varying extents across a wide variety of cancers, with both clonal and subclonal driver mutations identified. However, the majority of studies considering heterogeneity in detail have either been limited to a small number of patients or only investigated heterogeneity based on one sample from each tumor, thereby potentially underestimating the true extent of diversity within tumors.

BIOINFORMATIC TOOLS TO EXPLORE HETEROGENEITY

Data available from next-generation sequencing experiments are suited to statistical analysis to assess diversity within tumors given that sequencing data represent a random sample of DNA molecules, and by extension cancer cell genomes, within a given tumor cell population. As such, the advent of next-generation sequencing has seen a surge in computational tools to explore the clonal architecture of tumors, from both single and multiregion studies.

The fraction of reads reporting a point mutation in a sample is dependent on the copy number at that locus, the level of tumor purity (i.e., what proportion of cells that are sequenced are tumor

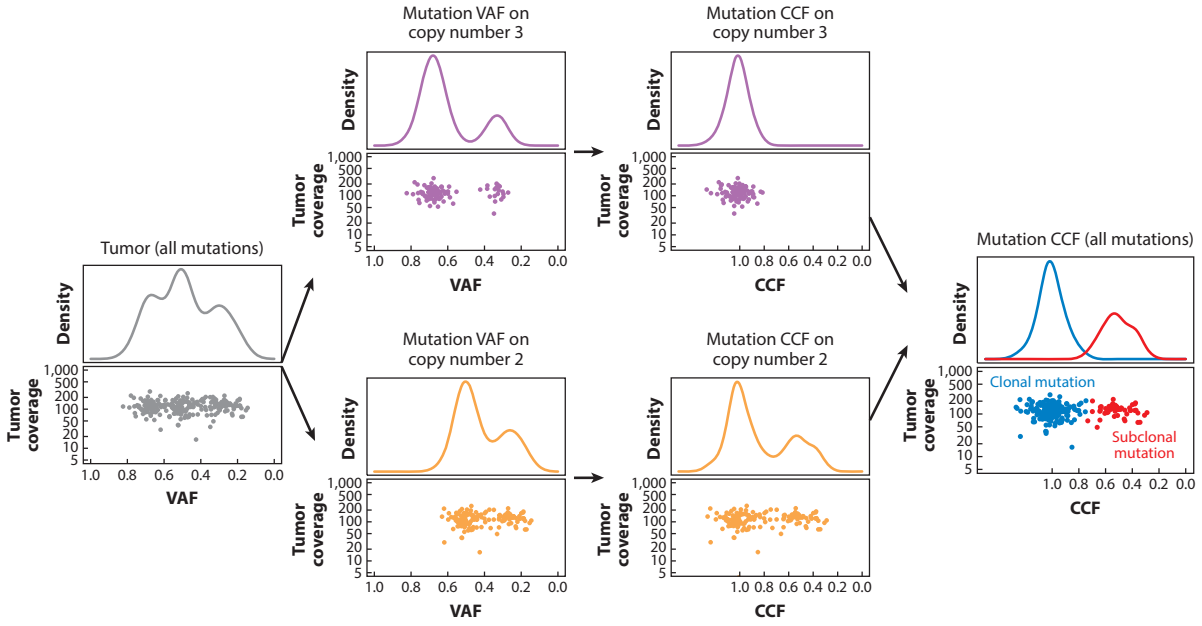


Figure 1

Correcting for copy number to determine the cancer cell fraction (CCF). The variant allele frequency (VAF) of each mutation will depend on its local copy number. By correcting for mutation copy number and then clustering mutations, one can determine clonal and subclonal mutations. In this hypothetical example, mutations occur in regions of the genome of copy number 2 (*orange*) or copy number 3 (*purple*). The distribution of VAF in regions of copy number 3 is consistent with all cells harboring these mutations, some on one copy and some on two copies of the three alleles present. On the contrary, the distribution of VAF in regions of copy number 2 shows that some of these mutations are subclonal.

cells), and finally the cancer cell fraction, describing the fraction of cancer cells in the sample that harbor the mutation. The majority of tools to dissect clonal architecture rely on the relationship between these variables, to estimate whether mutations are likely clonal or subclonal (**Figure 1**).

In general, a first step in dissecting the clonal architecture of a tumor involves estimating its genomic copy number profile and also its purity. Tools such as ASCAT (Van Loo et al. 2010), ABSOLUTE (Carter et al. 2012), OncoSNP (Yau et al. 2010), PICNIC (Greenman et al. 2010), and Sequenza (Favero et al. 2015) utilize mathematical frameworks to decipher copy number and data purity by assuming that sequenced DNA represents a mixture of measurements from a population of at least two distinct cell types present at different proportions: (*a*) tumors cells that contain an unknown amount of DNA and (*b*) an unknown proportion of normal cells with a known amount of DNA per cell. Although the system of equations is undetermined, only a few combinations of purity and ploidy can result in biologically meaningful solutions (Van Loo & Campbell 2012).

Once the copy number and purity of a sample have been determined, the cancer cell fraction of each mutation can be estimated. A simple approach to assess the clonality of a given mutation is to determine whether the observed variant allele frequency differs from what would be expected given a clonal mutation (Carter et al. 2012, Stephens et al. 2012). More sophisticated methods rely on the assumption that mutations with similar variant allele frequencies may correspond to clonal or subclonal clusters, reflecting nodes on an evolutionary tree. For example, PyClone (Roth et al. 2014, Shah et al. 2012) integrates variant allele frequencies with allele-specific integer copy

number estimates to define the subclonal composition within individual biopsies. The method uses a Bayesian Dirichlet clustering process to jointly group mutations, and infers posterior density estimates over the cancer cell fraction for each mutation. Conveniently, modeling the number of subclones as coming from a Dirichlet process does not require knowledge of the number of subclones a priori (Roth et al. 2014). PyClone assumes all copy number events are clonal, and deep sequencing is recommended (Roth et al. 2014).

The method adopted by Nik-Zainal et al. (2012b) leverages data from whole genome sequencing to circumvent the need for deep sequencing and allows mutations to reside on subclonal copy numbers. By contrast, SciClone (Miller et al. 2014), which also applies a Bayesian clustering method, focuses on single-nucleotide variants in balanced copy number regions. Although this feature of SciClone circumvents issues associated with clonal and subclonal copy number aberrations, it means that the clonality of every mutation cannot be determined.

More recent methods also use the fact that the structure of mutations should be hierarchical, with nested subclones (Deshwar et al. 2015, Jiao et al. 2014), as well as modeling different forms of data together (El-Kebir et al 2016, Fischer et al. 2014). These methods have also been extended to allow multiple samples over space or time to be included in subclonal clustering, and this may considerably improve the accuracy of subclonal reconstruction.

Importantly, it may be difficult to accurately deconvolve the subclonal structure in a tumor from a single sample. For example, if two subclonal populations have similar cancer cell fractions in one tumor region, they will appear as one clone. Analysis of another tumor region may enable their separation. Similarly, without single-cell or multiregion sequencing, an amplification to eight copies occurring in half the tumor population may appear like an amplification to four copies in the tumor population as a whole. In addition to multiregion sequencing, further information regarding the clonal composition of tumors can be inferred based on the mutual exclusivity or co-occurrence of mutations in cancer cells, either from single-cell sequencing or from phasing. Phasing involves determining whether mutations co-occur or are mutually exclusive, allowing different subclones to be delineated; if two mutations never appear to occur together on the same haplotype, they are likely to represent distinct subclones, whereas if two mutations can be phased in the same cancer cell they are necessarily present in the same lineage (Nik-Zainal et al. 2012b). However, phasing approaches are currently limited to the analysis of mutations in regions of hypermutation or high mutation burden.

Finally, it is important to recognize that the cancer cell fraction of a mutation need not directly reflect its timing or phylogenetic relationship with other mutations. For instance, given the dynamic nature of tumor evolution, an early truncal mutation may later be lost as a result of copy number alterations (McPherson et al 2016, Murugaesu et al. 2015). Equally, the identification of a mutation in every tumor region does not mean that it is necessarily truncal as subclonal mutations may be present at low frequencies in multiple tumor regions.

CANCER, SELECTION, AND NEUTRAL EVOLUTION

First formally proposed as such by Nowell (1976), tumor progression represents an evolutionary process under continuous Darwinian selection (Greaves & Maley 2012). At the time of detection, a tumor will have undergone many rounds of cell division, with each generation of cells stochastically acquiring novel somatic mutations (Gerlinger et al. 2014b). Although most of these mutations may have little impact on the overall fitness of the cell, a minority subset of these mutations (known as driver events) may endow a cell with an evolutionary advantage, allowing that cell and its progeny to flourish and outcompete others. Combined, the processes of clonal selection and clonal evolution acting on the cell may result in the outgrowth of multiple subclones, often with their own distinct

driver events, leading to the branched evolutionary phylogeny that has been observed across many cancer types (de Bruin et al. 2014, Gerlinger et al. 2012, Gundem et al. 2015, Nik-Zainal et al. 2012b, Sottoriva et al. 2013) (**Figure 2*a***).

Generally, the number of driver events required for tumor initiation and progression, and how this changes with time and the tumor microenvironment, remains unknown. Conventional theory has viewed tumorigenesis as a slow process of clonal evolution as driver mutations are gradually acquired, enabling various populations to expand, resulting in the formation of progressively more disordered clones (Merlo et al. 2006, Stratton et al. 2009). However, recent studies have shown that the malignant transformation of normal mammary tissue may also be induced surprisingly quickly and efficiently, requiring the use of only KRAS G12D as an oncogene (Nguyen et al. 2015). Additionally, the analysis of tumor genomes has recently revealed novel catastrophic events such as chromothripsis, chromoplexy, and kataegis, which may result in a drastic shift in the evolutionary trajectory of a tumor (Baca et al. 2013, Campbell et al. 2010, Forment et al. 2012, Nik-Zainal et al. 2012a, Shen 2013, Stephens et al. 2012).

Although the majority of established driver events are clonal, indicating that they likely arise early in tumor evolution, subclonal driver events have been identified across many cancers and are thought to play a role in tumor maintenance and progression, potentially leading to subclonal expansions (McGranahan & Swanton 2015). Even the most common driver events may occur early in some tumors while occurring late in others (McGranahan et al. 2015, Yates et al. 2015). This reflects that the delineation between driver and passenger mutations is context dependent. As selective pressures and the tumor microenvironment change, so do the requirements for tumor survival.

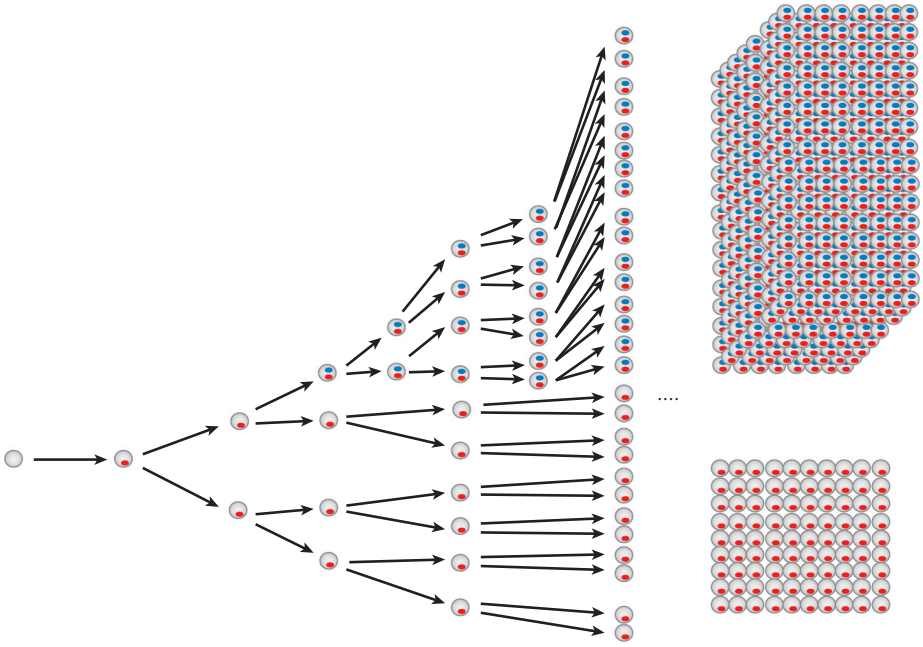
Subclonal populations of cancerous cells give rise to a heterogeneous environment within the tumor; however, each subclone is not an isolated entity. Interaction between subclones during tumor evolution may be competitive or cooperative. One subclone may outcompete another for vital resources, such as oxygen, nutrients, or space (Marusyk & Polyak 2010). Over time, different subclones may even alternate as the dominant clone in a population, indicating the dynamic selective pressures influencing clonal competition (Egan et al. 2012, Keats et al. 2012).

Alternatively, low-frequency clones may support the growth of the dominant clone or promote resistance to therapy through paracrine signaling networks (Hobor et al. 2014, Inda et al. 2010). Additionally, crosstalk between different cell populations is capable of shaping tumor properties, such as metastatic potential, as has been shown in mouse models of small-cell lung carcinoma (Calbo et al. 2011). It is not necessary for a subclone to have an obvious fitness advantage itself in order to affect tumor development, as the subclone may still drive tumor growth by inducing favorable changes in the microenvironment (Marusyk et al. 2014). However, if the subclone responsible for contributing to the advantageous growth conditions is outcompeted by another faster proliferating subclone, which itself is dependent on the current microenvironment, tumor collapse may occur (Maley et al. 2004, Marusyk et al. 2014).

In addition to the ongoing clonal selection evident in some cancers, recent work has also highlighted the occurrence of neutral evolution during tumor development (**Figure 2*b***). Using a theoretical model that determines the expected distribution of subclonal mutations under neutral evolutionary processes, Williams and colleagues (2016) demonstrated that the extensive heterogeneity observed in some tumors can be explained by neutral expansion and the accumulation of passenger mutations after any early driver mutations required for tumor initiation have been acquired. The absence of selective sweeps as predicted under a model of neutral evolution implies that once a mutation has arisen in a surviving lineage, it will persist and expand at the same rate as any other mutation. Thus, the entire historical record of tumor evolution can be observed in the current tumor genome. The earliest mutations that initiated tumor growth would be present in all tumor cells, whereas more recently acquired mutations would be permanently constrained

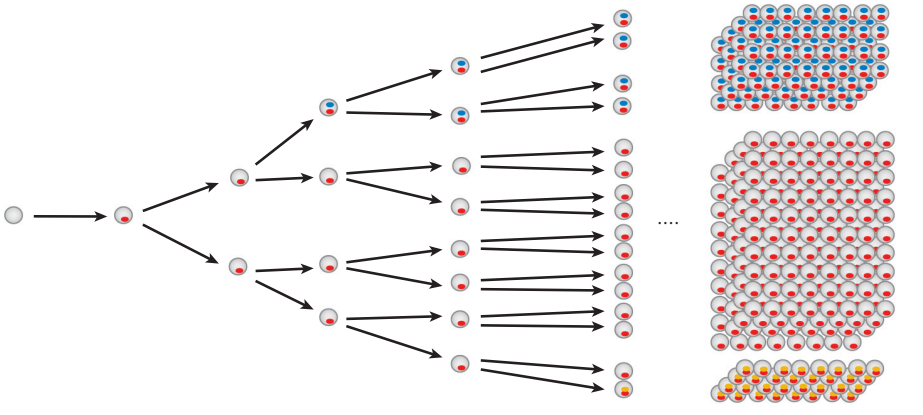
a Mutations under positive selection

Cellular frequency	0/1	1/1	2/2	4/4	5/5	10/10	14/14	28/28	1,120/1,120
VAF	0.00	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Cellular frequency	0/1	0/1	0/2	1/4	2/5	4/10	8/14	16/28	1,024/1,120
VAF	0.00	0.00	0.00	0.13	0.20	0.20	0.29	0.29	0.46



b Mutations under neutral evolution

Cellular frequency	0/1	1/1	2/2	4/4	8/8	16/16	512/512
VAF	0.00	0.50	0.50	0.50	0.50	0.50	0.50
Cellular frequency	0/1	0/1	0/2	1/4	2/8	4/16	128/512
VAF	0.00	0.00	0.00	0.13	0.13	0.13	0.13
Cellular frequency	0/1	0/1	0/2	0/4	0/8	1/16	32/512
VAF	0.00	0.00	0.00	0.00	0.00	0.03	0.03



to a smaller tumor subpopulation, without any selective sweeps occurring to change their relative prevalence. One consequence of this model is that the malignant potential of a tumor may be determined very early in development (Sottoriva et al. 2015). Although the distribution of subclonal mutations fits the neutral evolution model for a subset of tumors across many cancer types, it remains to be determined how prevalent the phenomena is with more comprehensive sampling strategies and how copy number corrections of the variant allele frequencies impact the model.

SELECTION AND SOMATIC EVOLUTION IN NORMAL AND PRECANCEROUS CELLS

Stochastically acquired mutations in normal and precancerous tissues may be selected for, eventually resulting in the clinical presentation of a tumor. However, before that stage, the analysis of noncancerous samples can shed light on the earliest stages of tumor development. Studies focusing on Barrett's esophagus, a precursor lesion to esophageal adenocarcinoma (EAC), have revealed that it is more likely to progress to cancer if there is a higher degree of clonal diversity present (Maley et al. 2006, Merlo et al. 2010). Indeed, by the time EAC is clinically diagnosed, there has often been such evolution that the percentage of mutations shared between EAC and the adjacent segments of Barrett's esophagus can be less than 20% (Ross-Innes et al. 2015).

The order in which mutations arise can influence the outcome of subsequent selective pressures, restrict evolutionary paths (Papaemmanuil et al. 2013), and affect the clinical behavior of disease presentation as well as response to therapy (Ortmann et al. 2015). In a recent study on the evolution of melanoma from precursor lesions, a BRAF V600E mutation alone was found to be sufficient to form a nevus; however, precursor lesions with *NRAS* or alternative *BRAF* mutations also harbored additional oncogenic mutations. Moreover, different melanoma subtypes evolved through separate means (Shain et al. 2015).

Evidence of mutation acquisition and selection can also be observed in noncancerous cells. For instance, although human hematopoietic stem/progenitor cells divide very rarely, they still stochastically acquire mutations that may confer a slight selective growth advantage. In an analysis of germline reference blood samples taken from patients from The Cancer Genome Atlas (TCGA) diagnosed with many different solid cancer types, mutations associated with leukemia and/or lymphoma were identified. Importantly, none of these patients had any observable sign of hematological malignancies, and the fraction of patients harboring leukemia- and/or lymphoma-associated mutations increased with patient age. Thus these mutations appear to represent the earliest stages of clonal selection and hematopoietic expansion (Xie et al. 2014). Furthermore, apparently healthy individuals who carried driver mutations in their blood have been shown to be at a higher risk of developing blood cancers (Genovese et al. 2014, Jaiswal et al. 2014).

High numbers of mutations and extensive ITH have also been identified in normal skin tissue from the eyelids of middle-aged individuals. Positive selection of many known squamous skin cancer driver genes has been identified, with some clones acquiring multiple driver mutations without undergoing malignant transformation. This observation and the fact that, in most cases, mutations were detected only in a small fraction of the cells suggest that clonal outgrowth is not solely determined by driver gene acquisition or is somehow curtailed early on (Martincorena et al. 2015).

Figure 2

Modes of tumor evolution. (*a*) When cancer cells are subject to positive selection, their frequency may increase, meaning that the variant allele frequency (VAF) of a mutation will not remain constant over time. (*b*) During neutral evolution, the frequency of a cancer cell clone will directly reflect its timing, with the VAF remaining constant.

THERAPY, SELECTION, AND HETEROGENEITY

Longitudinal analyses of tumor samples have consistently identified shifts in the genomes of samples taken before and after treatment with chemotherapeutics (Ding et al. 2012, Johnson et al. 2014, Landau et al. 2013, Mullighan et al. 2008, Murugaesu et al. 2015, Schuh et al. 2012), indicating that cancer therapy can play a major role in altering the genomic landscape of a tumor. One way therapy may impact the evolution of a tumor is by directly acting as a mutagenic agent. In this case, the genotoxic effects of chemotherapy may be reflected by changes to the mutational landscape and spectra of the tumor. Consistent with this, an analysis of primary and relapsed acute myeloid leukemia samples revealed an increase in the transversion rate among relapse-specific mutations after cytotoxic therapy, indicating that at relapse chemotherapy had altered the mutational spectrum (Ding et al. 2012).

A multiregion exome sequencing analysis of a small cohort of four patients with EAC taken before and after treatment with a platinum-containing chemotherapy identified an increase in C > A transversions at CpC sites present in the postchemotherapy samples of patients with residual disease (Murugaesu et al. 2015). Mutations in this particular context had been previously identified in *Caenorhabditis elegans* treated with cisplatin, a platinum-based chemotherapeutic (Meier et al. 2014). The majority of the mutations observed in the platinum-associated mutational context by Murugaesu et al. were subclonal, consistent with those mutations occurring late in tumor evolution, as would be expected for chemotherapy-induced mutagenesis. Consistent with these findings, a larger study of 30 paired EACs sampled before and after neoadjuvant chemotherapy also found a significant increase in C > A transversions after treatment (Findlay et al. 2016). As the presence of residual disease indicates an incomplete response to chemotherapy, the observation that some drugs leave behind clear signs of mutagenic activity in subclonal populations highlights the need to better determine which patients are most likely to clinically benefit from therapy.

Conceivably, mutations generated from chemotherapeutics may not only leave scars in the genome, but may also directly contribute to disease progression (**Figure 3*a***). In a seminal study, Johnson and colleagues (2014) examined the mutational landscape of a cohort of initial low-grade gliomas and their recurrences and found that 6 out of 10 patients treated with temozolomide recurred as hypermutated high-grade gliomas, with the majority of newly acquired mutations occurring in a temozolomide-associated context. Additionally, within this mutational context, they identified driver mutations in the RB and Akt-mTOR pathways. Their finding highlights how chemotherapy-induced mutagenesis is not limited to solely aiding genetic diversification, but that it can also influence the evolutionary path taken by the tumor, resulting, in this case, in a tumor of higher histological grade with poor prognosis.

A subset of tumors from patients who have been treated with alkylating agents, such as temozolomide, exhibit a significant increase in overall mutation burden, consisting of primarily C > T transitions at CpC and CpT dinucleotide sites (Alexandrov et al. 2013, Johnson et al. 2014). The proposed mechanism for resistance and hypermutation is via a selective advantage for clones that acquired inactivating somatic mutations of *MSH6*, rendering them resistant to alkylating agents, yet left to undergo accelerated mutagenesis due to the lack of effective mismatch repair (Hunter et al. 2006).

Additionally, chemotherapy-induced mutagenesis may confound conclusions drawn from genomic analyses. Somatic mosaic protein truncating variants in PPM1D were originally identified as associated with germline predisposition to breast cancer and ovarian cancer in women (Ruark et al. 2013). However, further analysis has revealed that these variants are more commonly observed in postchemotherapy cases rather than pretreatment cases, suggesting that they are somatic mutations caused by treatment (Pharoah et al. 2016, Swisher et al. 2016).

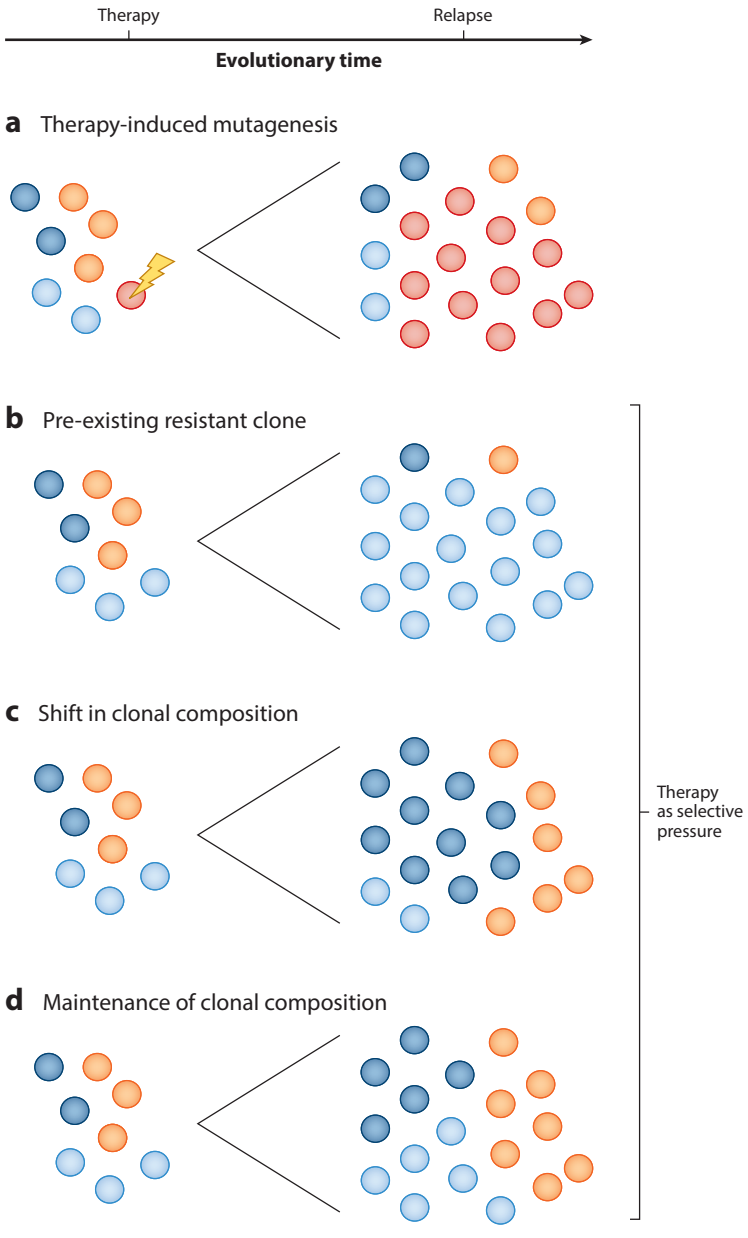


Figure 3

Modes of clonal evolution in response to therapy. Beginning with a heterogeneous population of cells, consisting of distinct subclones (*orange*, *light blue*, and *dark blue*), therapy can generate a mutation (*red*) that may (*a*) endow a subclonal population with a fitness advantage, (*b*) select for a pre-existing resistant clone, (*c*) result in clonal shifts without a known resistance mechanism, or (*d*) not substantially alter the clonal composition.

Even without directly inducing novel mutations, which may or may not be selected for, cancer therapy can alter the genomic landscape and heterogeneity of a tumor by applying new selective pressures, which can drive evolution reliant on the genetic variation that existed prior to the start of treatment. Within a heterogeneous tumor, some subclones may be present that originally had no obvious fitness advantage but impart a resistance to therapy and are subsequently selected for (**Figure 3*b***). Indeed, there have been numerous reports detailing the outgrowth of resistant subclonal populations in response to therapy across many cancer types, including colorectal cancer (Diaz et al. 2012, Kreso et al. 2013), glioblastoma (Cahill et al. 2007, Yip et al. 2009), melanoma (Shi et al. 2014, Wagle et al. 2011), NSCLC (Kosaka et al. 2006, Turke et al. 2010), and chronic myeloid leukemia (Shah et al. 2002).

Recent research has also shown the utility of sequencing circulating tumor DNA (ctDNA) taken at multiple time points to noninvasively monitor clonal dynamics and identify emerging resistance to therapy (Diaz et al. 2012; Murtaza et al. 2013, 2015; Russo et al. 2016). Murtaza and colleagues (2015) monitored a patient with metastatic ER-positive, HER2-positive breast cancer for more than 3 years and could infer the clonal evolution of the tumor over time in response to two separate classes of targeted therapy. They detected both the presence of a *PIK3CA* mutation at the time the patient progressed on trastuzumab and tamoxifen therapy and its decline after lapatinib treatment was begun. This was followed by an increase in allele frequency of an *ERBB4* mutation in response to lapatinib therapy that was predicted to contribute to the patient's resistance to the drug. Additionally, in colorectal cancer, integrative sequencing analyses of spatially and temporally separated tumor biopsies along with ctDNA have identified polyclonal mechanisms of resistance to EGFR blockade in distinct metastases from a single patient, highlighting the limits of relying on a single-lesion biopsy for decisions pertaining to the treatment of a heterogeneous tumor (Russo et al. 2016).

Importantly, although the emerging mutations identified on the path to drug resistance are often initially undetectable above background levels, little remains known about the evolution of resistant clones during the course of drug therapy. In particular, the sensitivity of next-generation sequencing is often not great enough to conclusively determine whether resistant clones existed before drug exposure, or if they evolved from the original clones to acquire resistance. In a recent study of *EGFR*-mutant NSCLC cell lines, Hata et al. (2016) used more sensitive sequencing technologies to track EGFR T790M drug-resistant clones and found that both the intrinsic and acquired routes to resistance could be observed. Intriguingly, they also found biological differences between those clones that had a pre-existing EGFR T790M mutation and those that were initially drug susceptible and evolved a T790M mutation de novo, which may help inform future therapeutic choices.

Clonal shifts without known resistance mechanisms have also been observed after treatment with cancer chemotherapy (**Figure 3*c***). Studies performed by Landau and colleagues (2013, 2015) in CLL have shown clear evidence of clonal evolution over time, where minor subclones at the start of treatment expand during the course of treatment. Often these subclones are enriched for driver mutations, suggesting that the selective pressure of treatment could remove incumbent clones and result in the growth of more aggressive subclones (Landau et al. 2013, 2015). Furthermore, they observed that the presence of a subclonal driver was independently associated with poorer outcome and response to therapy. Evidence of changes in clonal composition in response to therapy has also been detailed in multiple myeloma (Egan et al. 2012, Weston-Bell et al. 2013). Interestingly, Landau and colleagues (2013, 2015) also observed a minority of CLL cases that relapsed after undergoing treatment yet maintained a stable clonal equilibrium (**Figure 3*d***).

Examining the clonal dynamics of a tumor over multiple points in evolutionary time may also provide a means for future therapeutic intervention. A recent analysis of brain metastases and

their matched primary tumor samples observed that over half of the brain metastatic samples contained potentially clinically actionable alterations that were not ubiquitously present in the primary sample (Brastianos et al. 2015). Hata et al. (2016) also noted, with all the caveats of a limited sample size, that in their study of EGFR T790M mutant cell lines, the cell lines with a de novo *EGFR*-resistance mutation showed less evidence of a response to third-generation EGFR inhibitors, further highlighting the complexity of resistance mechanisms. Such observations could potentially have important implications in the clinic.

IMMUNE RESPONSE AND HETEROGENEITY

There is much evidence supporting an interaction between the tumor and the immune system (Dunn et al. 2002). Tumor cells may induce an immune response by expressing antigens that are recognized by a patient's T cells. Indeed, therapeutic tumor regression has been observed through the collection, ex vivo expansion, and re-administration of autologous tumor infiltrating lymphocytes resected from patients with metastatic melanoma. This indicates that there are T cells present capable of recognizing tumor cells and mounting an immune response against them (Dudley et al. 2002, Rosenberg 2012).

By recognizing some tumor-specific antigens, the immune system may also exert an evolutionary pressure, shaping the antigenicity of the tumor and its diversity as it evolves. Driven by the process of immunoediting, which describes the interaction between the tumor and immune system, in which the immune system plays the dual role of protecting the host and sculpting the tumor, subclonal populations of tumor cells either lacking immunogenic antigens or able to withstand an immune response may be selected for (DuPage et al. 2012, Matsushita et al. 2012, Schreiber et al. 2011).

Although an immune response may be mounted against self-antigens that are aberrantly expressed in cancerous tissues, recently there has been much interest in elucidating how the mutational landscape of a tumor may result in tumor-specific neoantigens that have arisen from nonsilent mutations. These neoantigenic mutations result in novel epitopes that may be recognized as foreign by a patient's tumor-infiltrating T lymphocytes (Rajasagi et al. 2014).

Through a pan-cancer study of patients from TCGA, Rooney and colleagues (2015) defined an expression-based measure of cytolytic activity and observed that it was positively correlated with the number of putative neoantigens in multiple tumor types. Furthermore, by comparing the observed and expected numbers of predicted neoantigens generated per nonsilent mutation, they found evidence of immunoediting, T cell–mediated surveillance, and elimination of subclones containing neoantigens. Additional studies of TCGA patients have shown that both the presence and number of predicted neoantigens are associated with overall survival (Brown et al. 2014, McGranahan et al. 2016).

Although the impact of the clonal architecture of the tumor on the neoantigen repertoire remains unclear (Heemskerk et al. 2013), there are some early indications that ITH might contribute toward shaping the immunogenicity of a tumor. It has been shown that, among nonhypermutated tumors, the most homogeneous tumors have the largest number of predicted neoantigens (Angelova et al. 2015, McGranahan et al. 2016). More heterogeneous tumors have been shown to have an overall greater depletion of immune subpopulations (Angelova et al. 2015) and lower levels of immune infiltration (Morris et al. 2016). Additionally, patients with homogeneous tumors exhibit longer overall survival than patients with more heterogeneous tumors (Angelova et al. 2015, McGranahan et al. 2016). These observations implicate ITH as a potentially important factor in determining the immune response elicited by a tumor.

IMMUNOTHERAPY AND HETEROGENEITY

The influence of ITH on the immune response may also have implications for personalized immune therapies, particularly those that target tumor neoantigens by modulating the activity of the immune system. The use of immune checkpoint inhibitors, such as antibodies directed against PD-1 (programmed cell death-1) or CTLA-4 (cytotoxic T lymphocyte-associated antigen-4), has shown great promise across a wide variety of cancers; however, clinical benefit has only been seen within a subset of the patient population (Hodi et al. 2010, Pardoll 2012, Topalian et al. 2012). To date, molecular determinants capable of predicting patient response to checkpoint blockade have been hard to establish. In seminal work studying NSCLC (Rizvi et al. 2015) and melanoma (Snyder et al. 2014, Van Allen et al. 2015), overall mutation burden has been shown to correlate with response to anti-PD-1 therapy and anti-CTLA-4 therapy, respectively, whereas in colorectal cancers (Le et al. 2015) mismatch repair status could predict clinical benefit to PD-1 blockade.

Based on the observations made in the treatment-naïve setting, it has been suggested that tumors with a low level of ITH and a high clonal neoantigen repertoire might further contribute to checkpoint inhibitor response. Conceivably, such patients may respond better because of the higher level of immune infiltration in more homogeneous tumors or because a highly homogeneous tumor itself may be evidence of extensive immunoediting of antigenic subclonal populations by a functional and active immune system (Angelova et al. 2015, McGranahan et al. 2016, Morris et al. 2016). Recent research further exploring the same checkpoint blockade–treated NSCLC and melanoma cohorts (Rizvi et al. 2015, Snyder et al. 2014, Van Allen et al. 2015) has found that response to PD-1 and CTLA-4 antibodies was particularly improved in patients with tumors enriched for clonal neoantigens. Among the patients treated with an anti-PD-1 antibody, those without durable benefit were found to have a significantly more heterogeneous neoantigen repertoire than those experiencing a durable clinical benefit. Additionally, incorporating a measure of heterogeneity, rather than considering total neoantigen burden alone, could more accurately stratify these patients into groups with durable clinical benefit or no clinical benefit (McGranahan et al. 2016).

These observations suggest that the clonal structure of neoantigenic mutations may play a role in immune surveillance and raise the question of whether subclonal mutations are sufficient to generate a tumor-wide immune response. Furthermore, among the anti-CTLA4-treated cohort, a small subset of patients previously treated with an alkylating agent had an increased mutational load of subclonal mutations, consistent with therapy-induced hypermutation, but were among the poor or limited responders, suggesting that although therapy may induce potentially immunogenic mutations, those might not always be sufficient to elicit an efficient antitumor immune response (McGranahan et al. 2016).

It is possible that in the presence of many antigenic subclonal mutations, an immune response is only mounted against a few of them, and even then, when lymphocytes are generated that recognize subclonal neoantigens, it is conceivable that they would not be able to target the whole tumor. Thus as more data become available, it will be important to determine the full extent of the interplay between the clonal architecture of a tumor and the potential reaction of the immune system.

FUTURE PERSPECTIVES

Above we consider the selective forces shaping a tumor as it develops, including the response to its environment and other extrinsic factors such as therapy. As the continuous acquisition of mutations results in a heterogeneous landscape that confers tumors with increased resilience, it will be of

particular importance to determine the impact the underlying clonal architecture has on tumor evolution. Most studies to date have been constrained by sequencing depth and single sample biopsies; therefore, calculations of tumor heterogeneity are bound to reflect underestimates.

Moving forward, improved computational methods to better dissect clonal architecture and model tumor clonal dynamics, as well as studies examining tumors with finer resolution through the use of deeper sequencing depth, single-cell samples, or multiple tumor regions, will help to elucidate the impact ITH has on a tumor's evolutionary path and potential. One such study has already been commenced, TRACERx [TRAcking Cancer Evolution through therapy (Rx)], which uses multiregion and longitudinal ultradeep exome sequencing to prospectively track the evolution of primary NSCLC from diagnosis through treatment and relapse (Jamal-Hanjani et al. 2014). Additionally, this study along with others has begun to analyze tumor immune infiltrates to assess how the immune system may shape tumor development and determine how heterogeneity impacts antitumor immunity both with and without the aid of immunotherapy (Angelova et al. 2015, Jamal-Hanjani et al. 2014, Llosa et al. 2015). Beyond genomic analyses of ITH, DNA methylation studies have also begun to identify extensive epigenetic ITH, adding yet another layer of complexity to the understanding of tumor evolution (Brocks et al. 2014, Mazor et al. 2015, Oakes et al. 2014).

Continuing such studies will lead to an improved understanding of ITH and clonal dynamics, endowing us with the ability to more fully decipher the evolutionary history of a tumor as well as a greater understanding of which events are truly clonal and how a tumor may respond to therapy. This will potentially translate into novel therapeutic approaches and inform new ways to best stratify patient groups for maximal treatment efficacy.

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