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Perspective

Biological and Therapeutic Impact of Intratumor Heterogeneity in Cancer Evolution

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Precision medicine requires an understanding of cancer genes and mutational processes, as well as an appreciation of the extent to which these are found heterogeneously in cancer cells during tumor evolution. Here, we explore the processes shaping the cancer genome, placing these within the context of tumor evolution and their impact on intratumor heterogeneity and drug development. We review evidence for constraints and contingencies to tumor evolution and highlight the clinical implications of diversity within tumors. We outline the limitations of genome-driven targeted therapies and explore future strategies, including immune and adaptive approaches, to address this therapeutic challenge.

Given the size of the human diploid genome (~6 billion base pairs), even without an elevated mutation rate, the potential for the acquisition of mutations over the course of a human lifetime is vast (Lynch, 2010). Tumors sequenced at the exome level have been found to harbor anything from merely one or two to thousands of somatic aberrations, ranging from base-pair substitutions to whole-genome doublings.

Tumors accumulate somatic aberrations through an evolutionary process (Nowell, 1976). While the majority of these aberrations are likely to be passenger events that do not provide any selective benefit to the cancer cell, a small subset will represent cancer driver events, conferring a selective advantage (Kandoth et al., 2013; Lawrence et al., 2014). Accumulating evidence suggests that not every mutation, whether driver or passenger, will be found in every cancer cell within a tumor (see reviews Swanton, 2012; Yates and Campbell, 2012). While the types and distribution of mutations across the genome in a cancer cell can be used to decipher the mutational processes that have been active during its evolutionary history (Helleday et al., 2014), the extent of heterogeneity and its dynamics over time can reveal a tumor's life history (Burrell et al., 2013a; Yates and Campbell, 2012).

The heterogeneity observed within tumors and the myriad of genome instability processes that shape tumor evolution over space and time have important clinical implications and may reflect the mismatch between cost and benefit of some anticancer therapies (Fojo et al., 2014). For instance, between 2002 and 2012, of 71 anticancer drugs approved by the Food and Drug Administration, including 52 targeted medicines, the median overall survival benefit was 2.1 months, balanced against an estimated $10,000 per month on therapy at a cost of $2.7 million per life year saved (Kantarjian and Zwelling, 2013). Targeted therapies will likely only have maximal efficacy when targeting somatic events present in all cancer cells and may be complicated by evidence that the number of cancer drivers in advanced tumors may be substantial (Gerlinger et al., 2014). Moreover, increasing evidence is emerging for the presence of polygenic drug-resistance mechanisms in subclones prior to the initiation of therapy (Bozic et al., 2013) and that low-frequency subclones can support the growth of the dominant clone. Future drug development strategies must therefore take into account clonal heterogeneity, as well as evidence that subclones can compete and synergize for growth in a symbiotic manner.

In this review, we explore the processes shaping the cancer genome and place these in the context of intratumor heterogeneity. We review the extent to which rules for tumor evolution, which may guide precision medicine, can be deciphered and outline the clinical implications associated with diversity within tumors. Finally, we explore strategies that could be adopted to help address this therapeutic challenge.

Biological Basis of Intratumor Heterogeneity and Cancer Evolution

Genomic Instability and Endogenous and Exogenous Mutational Processes

Genome instability processes result in an elevated rate of somatic aberrations, ranging from point mutations to chromosomal and whole-genome doublings. This instability can contribute to intratumor heterogeneity by providing a pool of mutations upon which selection can act in a given microenvironmental context (Burrell et al., 2013a). Thus, an understanding of genome instability processes is required to understand a biological basis for tumor heterogeneity.

The characteristic mutations associated with a particular genome instability process can be considered a "mutational signature," reflecting the imprint of the type of DNA damage that has occurred. Such mutational signatures may exist at both the nucleotide and chromosomal level simultaneously. For example, non-small-cell lung tumors (NSCLCs) from heavy cigarette smokers display a preponderance of C > A transversions and significantly more copy number gains and mutations compared with nonsmokers (Govindan et al., 2012; Huang et al., 2011; Pleasance et al., 2010), while colorectal cancers with endogenous mismatch repair deficiency exhibit an enrichment of C > T transitions, particularly at CpG sites, and generally show low levels of chromosomal alterations.

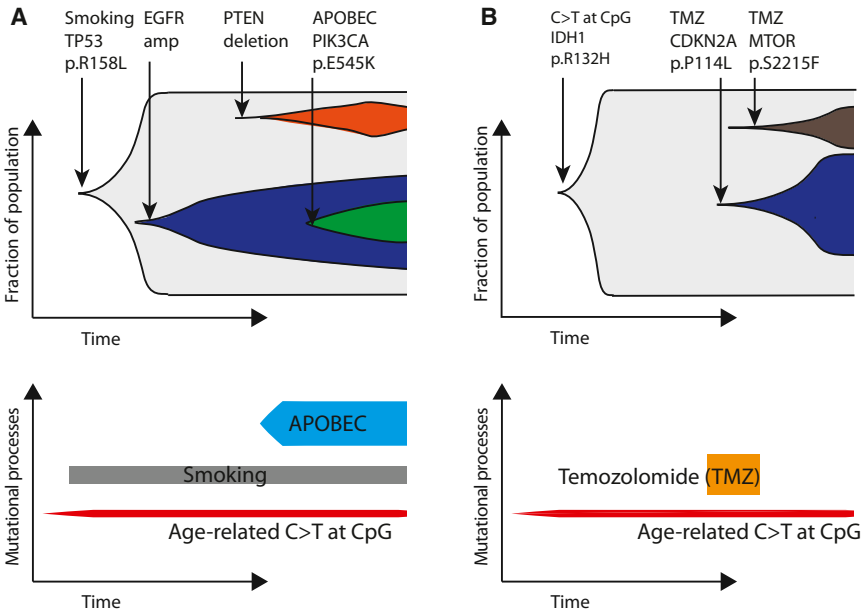

Figure 1. Endogenous and Exogenous Mutational Processes Alter the Evolutionary Trajectory of a Tumor

(A) An age-related mutational process operates throughout the evolution of a lung tumor. A smoking-induced C > A mutation in TP53 (p.R158L) leads to the outgrowth of a major tumor clone. Later in tumor evolution, APOBEC-mediated mutagenesis results in a mutation to PIK3CA (p.E545K), which leads to a subclonal expansion. (B) The evolution a glioblastoma tumor that has undergone treatment with Temozolomide (TMZ). Notably, TMZ leads to mutations in *CDKN2A* and *RB1* in separate subclones, both of which lead to subclonal expansions.

Genome Instability Processes at the Single Nucleotide Level

Recently, mathematical frameworks have been developed to quantify the number and contributions of mutational signatures operating within cancers at the single-nucleotide level (Alexandrov et al., 2013; Fischer et al., 2013). Application of nonnegative matrix factorization to more than 7,000 tumors from over 30 cancer types identified 20 distinct mutational signatures (Alexandrov et al., 2013). The plethora of mutational signatures identified reflects the diverse array of endogenous and exogenous genome instability processes that can operate in cancers during evolution. Intriguingly, in many cases, the underlying etiology of these mutational signatures remains unknown (Helleday et al., 2014).

In the majority of cancer samples analyzed, at least two mutational processes were identified, consistent with an elevated mutation rate in most cancers (Alexandrov et al., 2013). The most widespread mutational signature, identified in 25 cancer types, was characterized by C > T transitions at CpG sites, probably reflecting deamination of 5-methylcytosines at CpG sites. This signature correlated with patient age (Alexandrov et al., 2013), consistent with a large proportion of these mutations having been acquired prior to tumorigenesis.

Another pervasive mutational signature, identified in 15 cancer types, has been linked to the endogenous activity of apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) cytidine deaminases and is characterized by C > T and C > G mutations at TpC sites (Alexandrov et al., 2013; Burns et al., 2013; Roberts et al., 2013). APOBEC-mediated mutagenesis can also be linked to the acquisition of driver mutations, emphasizing the likely importance of this mutational process in the shaping of the evolution of the cancer genome (Figure 1A). A large proportion of *PIK3CA* helical domain mutations in human papillomavirus-driven tumors display an APOBEC motif (Henderson et al., 2014). Moreover, in NSCLC, our group found evidence that while early mutations were dominated by smoking induced C > A transversions, APOBEC-mediated mutagenesis was the dominant mutational force later in tumor evolution, conceivably providing a fertile substrate for tumor adaptation to environmental and targeted, cytotoxic, or radiation therapy-induced selection pressures (de Bruin et al., 2014). Consistent with the importance of this mutational process later in NSCLC evolution, over 15% subclonal mutations in driver genes, including *PIK3CA*, *TGFBR1*, and *PTPRD*, were found within an APOBEC context. Additionally, geographically distinct regions of the same tumor displayed different levels of APOBEC-mediated mutagenesis (de Bruin et al., 2014), suggesting drivers of diversity themselves can be both spatially heterogeneous and alter in dominance over time.

Therapy may also act as an exogenous source of genome instability (Cahill et al., 2007; Ding et al., 2012; Hunter et al., 2006; Johnson et al., 2014). Ding et al. (2012) studied the clonal evolution of primary and relapsed acute myeloid leukemia (AML) and found an increase in transversions following cytotoxic therapy at relapse (46%) compared with mutations prior to therapy (30.7%). Consistent with this, in *C. elegans*, cisplatin treatment has been found to lead to a striking, dose-dependent increase in base substitutions—predominantly C > A transversions—as well as an elevated rate of dinucleotide substitutions, indels, and structural variants (Meier et al., 2014). Temozolomide treatment has also been found to leave an imprint in the cancer genome in the form of an elevated rate of C > T transitions—primarily at CpC and CpT sites (Alexandrov et al., 2013; Johnson et al., 2014). Costello's laboratory performed a comparison of the genomic landscape of gliomas at initial diagnosis and recurrence and found that 6 of 10 tumors that recurred as glioblastomas, a high-grade tumor with worse prognosis, displayed evidence of hypermutation—exhibiting 7–450 times the mutational load per megabase compared with primary gliomas. Furthermore, all hypermutant tumors were treated with temozolomide, and many temozolomide-induced mutations were found in driver genes, including *RB1* and *CDKN2A* (Johnson et al., 2014) (Figure 1B). In these examples, therapy was not acting merely as an exogenous source of mutations but also as a selection barrier, shaping the evolutionary trajectory of a tumor and its progression to a more aggressive phase.

Consistent with therapy acting as a selection barrier, in NSCLC, chemotherapy was found to reduce *EGFR* mutation

frequency (Bai et al., 2012), and treatment of colorectal cancer clones with oxaliplatin resulted in outgrowth of previously dormant, resting clones (Kreso et al., 2013). In this case, chemotherapy did not act as an exogenous source of mutations, as the effect was independent of acquired genetic mutations, highlighting the importance of nongenetic mechanisms in generating diversity within tumors. In support of this, phenotypic behavior and fate of identical daughter cancer cells can be vastly different upon treatment despite identical genetic backgrounds (Gascoigne and Taylor, 2008). Interestingly, in AML, while morphological and phenotypic features as well as growth properties were found to correlate with distinct genetically defined subclones, the engraftment of AML cells in mice did not relate to the genetically defined evolutionary hierarchy (Klco et al., 2014).

Genome Instability Processes at Copy Number Level

Genome instability processes and mutational signatures can also be deciphered through copy number analysis. Homologous recombination (HR) deficiency is thought to lead to a specific copy number profile, resulting in allelic imbalance (Abkevich et al., 2012; Birkbak et al., 2012; Popova et al., 2012). The clinical importance of this HR signature is underscored by the observations that it predicts cisplatin sensitivity in vitro and response to preoperative cisplatin treatment in patients with triple-negative breast cancer (Birkbak et al., 2012). Copy number aberrations can also be used to quantify the level of chromosomal instability (CIN) (Birkbak et al., 2011), a driving force of intercellular genetic heterogeneity (Lengauer et al., 1997). In colorectal cancer, aneuploid tumors frequently harbor loss of chromosome 18q. We have found that loss of three ''CIN-suppressor genes'' encoded on 18q is an early event in tumor evolution occurring at the onset of aneuploidy. Depletion of these three genes in vitro initiates replication stress and generation of structural CIN and numerical CIN defined by centromeric fluorescence in situ hybridization, resulting in intercellular heterogeneity (Burrell et al., 2013b).

Chromothripsis, a single event that results in tens to thousands of chromosomal rearrangements localized to one or a few chromosomes (Stephens et al., 2011), is thought to occur in 5% of cancers and can be detected using allele-specific copy number data (Zack et al., 2013). The event likely results from distinct chromosomes or chromosomal regions becoming fragmented into multiple segments and then being pieced back together inaccurately through DNA repair mechanisms.

Finally, at the genome level, whole-genome doublings have been documented to occur frequently across a range of cancers and can be estimated from allele-specific copy number data (Carter et al., 2012; Dewhurst et al., 2014; Zack et al., 2013). Although the underlying causes and tolerance mechanisms of genome doubling remain unclear, it has been postulated to represent a macroevolutionary leap in the development of tumors. This is supported by observations that genome doubling is associated with accelerated cancer genome evolution and elevated levels of chromosomal alterations (Dewhurst et al., 2014; Zack et al., 2013).

Contingency, Convergence, and Rules of Evolution

While analysis of individual cancer genomes can shed light on the mutational processes that have been operative during tumor evolution, from a therapeutic perspective, there is a need to determine whether trends and patterns in the evolution of cancer genomes through space and time can be deciphered. This issue is reminiscent of the long-standing and contentious debate on whether macroevolutionary trends and rules exist and Gould's famous assertion that if the tape of life were rewound and played again a different evolutionary outcome would result (Gould, 1989). Such a notion does not imply evolution is random; rather, the final outcome is contingent upon the sequence of antecedent steps (Gould, 1989). Convergence, on the hand, has been championed as an opposing theory to contingency, suggesting that constraints to evolution may lead to a limited set of potentially repeated outcomes. Studies exploring evolutionary histories of tumors and epistatic interactions have begun to shed light on the interplay between contingency and convergence in cancer development and the possibility of an evolutionary rulebook dictating cancer evolutionary routes (Ashworth et al., 2011).

Modes of Tumor Evolution and Cooperation between Tumor Subclones

Longitudinal (Johnson et al., 2014; Mullighan et al., 2008; Shah et al., 2009), spatial (Aerts et al., 2014; Bashashati et al., 2013; Campbell et al., 2010; Gerlinger et al., 2012, 2014; Haffner et al., 2013; Navin et al., 2010, 2011; Thirlwell et al., 2010; Yachida et al., 2010), and in-depth mapping of single tumor samples (Anderson et al., 2011; Nik-Zainal et al., 2012a, 2012b; Shah et al., 2012) are increasingly revealing a process of branched tumor evolution across multiple cancer types (Figure 2; for reviews, see Navin, 2014; Swanton, 2012; Yates and Campbell, 2012).

Recent studies have also shed light on the extent to which genetically distinct subclones interact during tumor evolution (Calbo et al., 2011; Inda et al., 2010; Marusyk et al., 2014; Misale et al., 2012). In glioblastoma, a low-frequency EGFRvIII subclone was found to contribute to growth of the dominant clone through paracrine mechanisms (Inda et al., 2010). Co-operation of clones has also been documented in mouse Wnt-driven mammary tumors (Cleary et al., 2014) and *Drosophila*, where distinct clones bearing RAS^{V12} and *SCRIB* loss of function mutations cooperate to induce JNK signaling and activation of growth promoting cytokines (Wu et al., 2010). Likewise, using a zebrafish melanoma xenograft model, inherently invasive as well as poorly invasive melanoma subpopulations can coinvade in a symbiotic manner, without clonal selection or phenotype switching (Chapman et al., 2014).

Conceivably, clonal cooperation applies to many aspects of tumor growth and progression. Indeed, in a mouse model of small-cell lung cancer, Berns and colleagues (Calbo et al., 2011) demonstrated cross-talk between two histopathologically distinct populations of neuroendocrine and mesenchymal cells sharing the same genetic origin (Calbo et al., 2011). The neuroendocrine cells acquired metastatic potential when the two cellular populations were engrafted together (Calbo et al., 2011). Relatedly, in colorectal cancer Alberto Bardelli's group recently demonstrated that low-frequency *KRAS* mutant subclones—that are resistant to cetuximab—can support the survival of *KRAS* WT, drug-sensitive subclones through the paracrine release of transforming growth factor β and amphiregulin (Hobor et al., 2014). Clonal cooperation may explain observed clonal equilibrium in chronic lymphocytic leukemia (CLL), in which the relative sizes of subclones were found to persist over several

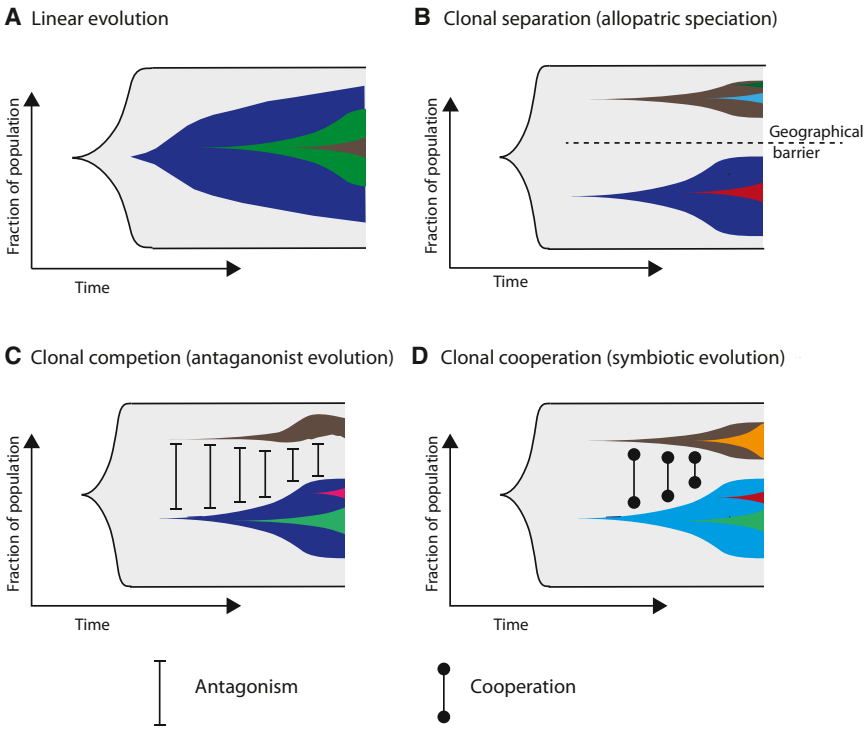

Figure 2. Modes of Tumor Evolution
(A) Linear evolution involves sequential accumulation of mutations over time. As can be seen, linear evolution can result in heterogeneity if a subclone has failed to outcompete its predecessors.
(B) Tumor subclones may evolve through a process equivalent to allopatric speciation when subclonal populations are geographically distinct within a tumor.
(C) Clonal competition can occur between subclonal populations, where distinct subclones compete for growth advantages (equivalent to an antagonistic relationship).
(D) Subclonal populations may cooperate, resulting in a symbiotic relationship.

years (Landau et al., 2013). However, it is also worth noting that clonal cooperation can result in tumor collapse through clonal interference; for example, if the non-cell-autonomous driver subclone is outcompeted by a subclone with higher proliferative potential that cannot survive independently (Marusyk et al., 2014).

Taken together, these data suggest that tumors represent a complex dynamic ecological system where heterogeneity is not only a substrate for evolution, but can also promote, or even be a requirement for, continued tumor development and progression. It will be important to determine the extent to which subclones compete and cooperate in different tumor types and in individual cases. In NSCLC and clear cell renal cell carcinoma (ccRCC), tumor subclones within the primary tumor appear geographically distinct (de Bruin et al., 2014; Gerlinger et al., 2014). These observations suggest a process equivalent to allopatric speciation might be operating in these tumor types, whereby subclones become geographically isolated, resulting in genetically distinct subclones in different tumor regions (Figure 2B). Nevertheless, evidence that subclones can compete and synergize for growth suggests in certain cases that cancer drug development strategies may have to adapt to identify and target small populations of cells that support the growth and survival of neighboring cells in the tumor.

Temporal Dissection of Mutations and Epistatic Interactions

From a clinical standpoint, if mutations in certain genes are always early events, these may be particularly appealing targets for therapy (Table 1). In colorectal cancer, Fearon and Vogelstein (1990) used the frequency of somatic events across independent colorectal tumors at different stages of tumor development to infer their likely temporal order. According to this model, there are two routes resulting in colorectal cancer, one through inactivation of *APC* and the other through mismatch repair deficiency. Such a model becomes more complex when branched evolution is considered within tumors. In fact, heterogeneity itself can be used to infer the temporal sequence of somatic events in cancer. Clonal mutations, occurring on the trunk of a tumor's phylogenetic tree, are early events, whereas subclonal events, occurring on the branches, reflect later events (Campbell et al., 2010; Gerlinger et al., 2012; Greaves and Maley, 2012; Landau et al., 2013). In addition, computational methods have been developed to elucidate the temporal acquisition of genomic events in cancers from cross-sectional mutation data (Attolini et al., 2010; Beerenwinkel et al., 2014; Gerstung et al., 2011).

Multiregion sequencing of 10 ccRCCs has revealed that mutations in von Hippel-Lindau gene (VHL), together with loss of chromosome 3p, are obligatory early events in this cancer type (Gerlinger et al., 2014). However, mutations in *TP53*, *SETD2*, *BAP1*, *PTEN*, *MTOR*, and *KDM5C* were only ever found to be subclonal, suggesting these are later events in ccRCC evolution. In contrast to ccRCC, in breast, ovarian, pancreatic, and esophageal cancers, *TP53* mutations have been found generally to be early events (Bashashati et al., 2013; Nik-Zainal et al., 2012b; Shah et al., 2012; Weaver et al., 2014; Yachida et al., 2010). Indeed, *TP53* was found to be one of the only mutations that could predict progression from Barrett's esophagus to esophageal adenocarcinoma (Weaver et al., 2014). This implies that in many cancers mutations in *TP53* may be one of the founder mutations, while in other cancers *TP53* mutations may play a role in maintenance and progression, occurring at the onset or after subclonal diversification. In colorectal cancer, driver mutations in *KRAS*, *NRAS*, and *BRAF* were found to be concordant between primary tumor and metastasis, implying these are often early events (Brannon et al., 2014). In contrast, in myelodysplastic syndrome (MDS), it was found that mutations in *NRAS* were among the latest events while mutations in genes involved in splicing, such as *U2AF1*, were often the earliest (Papaemmanuil et al., 2013).

In the context of multiple myeloma (MM), driver events such as *BRAF* mutations can be clonal in some patients and subclonal in others, where they can co-occur with RAS mutations,

Table 1. Summary of Truncal and Branched Driver Events across Cancer Types

Tumor Type	Trunk Drivers[a]	Branch Drivers	References
AML	*DNMT3A*, *TET2*, t(15;17), t(8;21), t(16;16), inv(16)	*WT1*, *KRAS*, *NRAS*, *KIT*	Welch, 2014
Breast	*TP53*, *PIK3CA*	*BRCA2*	Martins et al., 2012; Nik-Zainal et al., 2012a; Shah et al., 2012
CLL	*MYD88*	*SF3B1*, *TP53*	Landau et al., 2013
Colorectal[b]	*KRAS*, *NRAS*, *BRAF*	*TP53*, *PIK3CA*	Brannon et al., 2014; Vakiani et al., 2012
Ewing Sarcoma	*EWSR1-ETS* fusion	*STAG2*	Tirode et al., 2014
Follicular lymphoma	BCl2-IGH (14;18), *MLL2*, *CREBBP*, *EZH2*	*MYD88*, *TNFAIP3*, *MYC*, *TP53*	Okosun et al., 2014
Glioma	*IDH1*	*SMARCA4*, *BRAF*, *TP53*, *ATRX*	Johnson et al., 2014
MDS	*SF3B1*, *SRSF2*, *U2AF1*, *DNMT3A*	*NRAS*	Papaemmanuil et al., 2013
Melanoma[c]	*BRAF*	*NRAS*, *MEK1*	Van Allen et al., 2014
Myeloma	*IgH* rearrangements	*KRAS*, *NRAS*, *BRAF*, *FAM46C*	Bolli et al., 2014; Lohr et al., 2014; Melchor et al., 2014
NSCLC	*BRAF*, *NF1*, *TP53*, *EGFR*	*HGF*, *MLL3*	Chen et al., 2012; de Bruin et al., 2014; Govindan et al., 2012
Esophageal adenocarcinoma	*TP53*, *SMAD4*	*MYO18B*, *TRIM58*, *CNTNAP5*, *ABCB1*, *PCDH9*, *UNC13C*, *SEMA5A*, *CCDC102B*	Weaver et al., 2014
Ovarian	*TP53*	*PIK3CA*, *CTNNB1*, *NF1*	Bashashati et al., 2013
Prostate	*ERG* rearrangements, 21q22 deletion, *NKX3-1* deletion *FOXP1*, *SPOP*	*PTEN*, *CDKN1B, AR* amplification	Baca et al., 2013; Haffner et al., 2013
Pancreatic	*KRAS*, *CDKN2A*, *TP53*, *SMAD4*	*OVCH1*	Yachida and Iacobuzio-Donahue, 2013
Renal	*VHL*, *PBRM1**, 3p loss of heterozygosity	*SETD2*, *BAP1*, *KDM5C, MTOR*, *TSC1*, *TSC2*, *TP53*	Gerlinger et al., 2012, 2014

[a]Genes with an asterisk have also been found to be subclonal in multiregion samples.
[b]Comparative sequencing analysis was used between matched primary and metastatic colorectal lesions to define potential branched status.
[c]Branched drivers defined in BRAF mutant melanoma.

suggesting these alterations can contribute to either tumor initiation or maintenance and progression (Bolli et al., 2014; Lohr et al., 2014). Similarly, in CLL, mutations in *TP53* were found to be subclonal in approximately 50% of cases and only mutations to *MYD88* were found to be almost always clonal (Landau et al., 2013). Evidence of subclonal driver mutations may complicate targeted therapy approaches (see below).

Epistatic interactions, whereby the action of each gene is dependent on its genetic background, may play a key role in dictating the order in which mutations are acquired (Figure 3A). For example, in the presence of WT p53, loss of *BRCA1* or *BRCA2* results in acute cell-cycle arrest; thus, it is likely that *TP53* mutations usually occur before BRCA loss of function (Ashworth et al., 2011). This scenario is supported by studies in BRCA1-associated breast tumors where loss of the remaining WT *BRCA1* often occurred after loss of *TP53* (Martins et al., 2012). Moreover, the early somatic alterations were found to influence the evolutionary trajectory of a tumor, with the majority of luminal breast tumors displaying early mutations to *TP53*, while loss of *PTEN* was observed as the first event in basal-like breast cancers. Similarly, in MDS, the type of early driver mutations can dictate the future trajectories of disease evolution (Papaemmanuil et al., 2013). These results suggest that certain mutations may result in a form of genetic canalization in which a tumor is forced down a particular evolutionary path in which subsequent evolutionary opportunities are restricted.

Parallel Evolution

In evolutionary biology, parallel evolution is defined as the development of similar traits in related but distinct species, descending from the same ancestor. Despite striking diversity within individual tumors, parallel evolution occurring in genetically distinct subclones is an emerging theme across multiple malignancies (Figure 3).

In acute lymphoblastic leukemia (ALL), deletions in *ETV6*, *PAX5*, and *CDKN2A* were found to occur independently in distinct subclones from the same tumor (Anderson et al., 2011). Similarly, in metastatic pancreatic cancer, distinct metastatic sites have been found to harbor independent out of frame deletions of exon 6 of *PARK2* (Campbell et al., 2010). In ccRCC, we found evidence of parallel evolution in six of ten tumors analyzed, with distinct somatic events in different tumor regions affecting the same gene (e.g., *SETD2*, *KDM5C*), pathway (*PIK3CA*, *PTEN*, *MTOR*) or protein complex (*PBRM1*, *ARID1A*, and *SMARCA4*) (Gerlinger et al., 2014). Moreover, by investigating four tumors occurring in the kidneys of a young patient with Von Hippel Lindau syndrome, we explored both contingencies and convergence during tumor evolution. Each tumor was found to have an independent clonal origin, with distinct chromosome 3p LOH events, resulting in biallelic inactivation of *VHL*. However, despite distinct tertiary driver events in every tumor, conceivably contingent on the prior 3p LOH event, convergence for functional activation of the mTOR pathway was observed in all four tumors (Fisher et al., 2014).

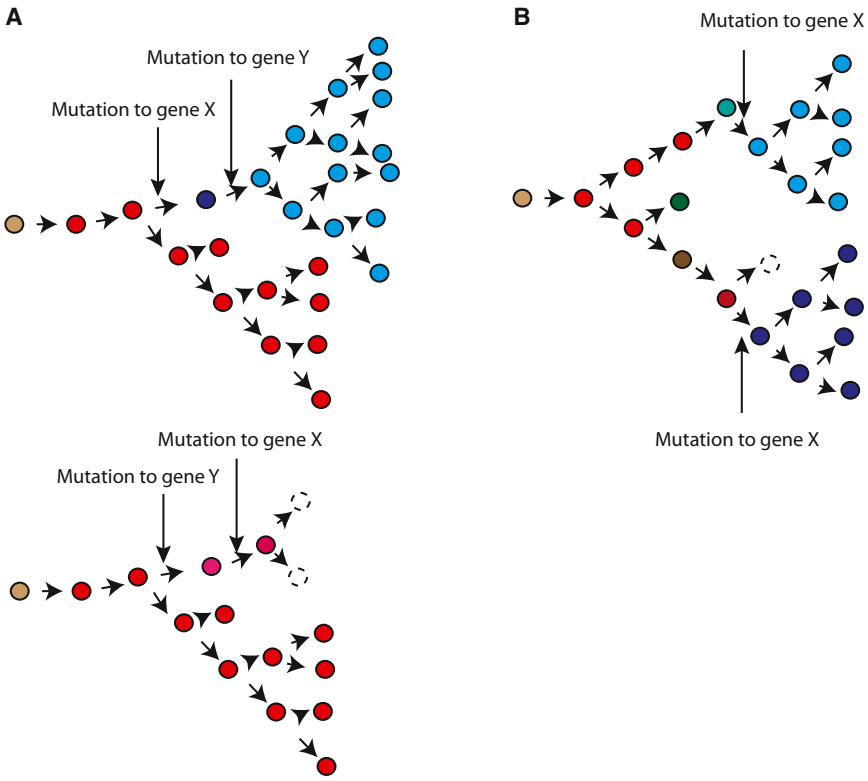

Figure 3. Epistatic Interactions and Parallel Evolution

(A) The order of mutational acquisition can influence the evolution of a tumor. If gene X is mutated before gene Y this results in a subclonal expansion, whereas if gene Y is mutated before gene X this results in cell death.

(B) Two subclonal populations of tumor cells independently acquire mutations to gene X, resulting in parallel evolution.

In recurrent glioma, mutations in *ATRX* and *TP53* distinct from mutations in the same genes identified in the primary tumor have been observed (Johnson et al., 2014). Similarly, in glioblastoma, through a single cell sequencing approach (leveraging data from bulk genomic sequencing), Francis et al. (2014) demonstrated parallel evolution of somatic alterations following *EGFR* amplification, involving distinct EGFRvII, EGFRvII-ext., *EGFR* (del25-28), and *EGFR* (del25-26) alterations in different cells within the same tumor. Distinct mutations in *KMT2D*, *TNFRSF14*, and *CREBBP* occurring independently in two patients with follicular lymphoma and their paired transformed follicular lymphomas, occurring later in the disease course, suggest critical dependencies on mutational events in these genes for tumor maintenance or progression (Okosun et al., 2014). Finally, in myeloma, independent subclones within the same tumor driving RAS/MAPK pathway activation through distinct *RAS* mutations have been observed (Melchor et al., 2014).

Evidence for parallel evolution and recurrent patterns in the temporal acquisition of mutations argues against viewing tumor evolution as a purely contingent process and emphasizes the existence of constraints to tumor development. Further clonal evolution studies in thousands of tumors together with a deeper understanding of the host microenvironment and germline may allow the prediction of future evolutionary paths and herald pre-emptive treatment strategies in contrast to current reactive clinical approaches.

Clinical Implications of Intratumor Heterogeneity

Drivers, Heterogeneity, and Outcome

While it has long been established that CIN, resulting in cell-to-cell genetic heterogeneity, is associated with poor prognosis across a wide range of cancers (McGranahan et al., 2012), the relationship between clonal heterogeneity and outcome and the clinical significance of subclonal driver mutations is only beginning to be explored across cancers.

In CLL, the presence of subclonal drivers was associated with a shorter time to retreatment or death (Landau et al., 2013), and in head-and-neck cancer, a measurement capturing the clonal diversity—termed mutant allele tumor heterogeneity—was found to correlate with poor prognosis (Mroz and Rocco, 2013). In 11 early-stage NSCLCs, primary tumors from three patients with relapsed disease had significantly larger subclonal fractions than tumors from patients with relapse free tumors (Zhang et al., 2014). In MDS, the number of driver events was the key determinant of outcome, regardless of their clonal status; i.e., the presence of a driver was more critical than whether it was subclonal or clonal (Papaemmanuil et al., 2013). Interestingly, in a series of 28 patients with MM, those with the highest cytogenetically defined clinical risk harbored disease that was most dynamic during the course of treatment (Keats et al., 2012). Likewise, in ccRCC, a poor prognosis ccB signature that remains a significant prognostic indicator in multivariate analysis defines tumors with multiple high-risk genetic alterations, potentially catalyzed by CIN (Gulati et al., 2014). Similarly, an exploration of glioblastoma tumors at the single-cell level revealed that proneural tumors harboring more diverse transcriptionally defined subtype repertoires were associated with poorest outcome (Patel et al., 2014).

Taken together, these data suggest that the plasticity of the cancer genome permits dynamic subclonal changes and the gain and loss of distinct genetic aberrations during the disease course. This plasticity may allow the tumor to adapt in the presence of microenvironmental pressures (Melchor et al., 2014). Although these studies hint at the clinical importance of intratumor heterogeneity, there is a need to prospectively explore the impact of plasticity and diversity within tumors and the relevance of subclonal driver events to therapeutic outcome. In NSCLC, a United Kingdom-based longitudinal study, Tracking Non-small Cell Lung Cancer Evolution Through Therapy (TRACERx http://clinicaltrials.gov/show/NCT01888601), has been launched to assess this (Jamal-Hanjani et al., 2014).

Targeted Therapy and Polygenic Drug Resistance

Most drug development programs employing next-generation sequencing as a stratification tool do not consider the clonal or subclonal frequencies of a driver alteration, simply their presence or absence. Indeed, major targeted therapy strategies are in progress targeting the PI3K signaling axis despite evidence that somatic mutations in members of this pathway, including *PTEN*, *PIK3CA*, and *MTOR*, are often or always subclonal in ccRCC, ovarian, and prostate cancers (Table 1). The clinical impact of driver variant allele frequency and the relative dominance of subclones with actionable alterations are priority areas for development within the context of clinical trial design.

Emerging patterns of the temporal acquisition of mutations should further inform targeted therapy approaches. Until we have a greater understanding of complex paracrine and non-cell-autonomous interactions of cancer subclones, targeting a clonally dominant, truncal driver may provide a more effective drug development strategy than simply considering actionable alterations as present or absent (Lohr et al., 2014; Yap et al., 2012). The DARWIN trial (Deciphering Anti-tumor Response With INtratumor Heterogeneity) (http://clinicaltrials.gov/show/NCT02183883) aims to assess whether targeting a clonally dominant driver event results in improved progression free survival outcomes relative to targeting the same driver event when it is present subclonally. In addition, these studies will monitor the subclonal dynamics through therapy and during the acquisition of drug resistance.

It is also important to consider the impact an emerging resistant subclone might have on disease biology in the face of continued drug exposure. In the majority of cases of BRAF mutant melanoma, resistance to a BRAF inhibitor is mediated by reactivation of the RAS-ERK signaling pathway, for example, through a mutation to RAS. Marais and colleagues recently demonstrated that continued BRAF inhibitor treatment in RAS and BRAF mutant melanoma cells results in release of proteases and a morphological switch that fosters tumor metastatic progression (Sanchez-Laorden et al., 2014). These data support the contention that rather than therapy having no effect on the behavior of drug resistant disease, in certain contexts, continued therapy in the presence of a resistant subclone might accelerate tumor progression. Likewise, evidence is emerging that in MM use of BRAF inhibitors in BRAF WT or RAS mutant clones results in paradoxical activation of the RAS-ERK pathway (Lohr et al., 2014).

These data raise caution when considering an actionable somatic event as either present or absent and suggest future drug development efforts will have to account for the clonal or subclonal nature of driver events prior to targeted intervention. Indeed, these complexities emphasize the continued need for in-depth biological understanding of drug response within the context of controlled clinical protocols. Ignoring the fact that driver events may be heterogeneous and thereby their potential deleterious impact in the face of therapeutic selection pressures, risks undermining a central principle of bioethics in medicine—"first do no harm."

Mechanisms of resistance may be driven by the presence of subclones barely detectable at presentation. Evidence in melanoma (Shi et al., 2014; Van Allen et al., 2014) and colorectal cancer (Diaz et al., 2012) suggests that resistance to therapy may occur through multiple somatic events simultaneously within the same tumor. For example, following BRAF inhibitor therapy in *BRAF* V600 mutant melanoma, individual tumors were found to develop multiple resistance events, including *NRAS* and *MEK1* mutations in one patient and two distinct *NRAS* mutations in another (Van Allen et al., 2014). Moreover, resistance to BRAF inhibitors can occur through both mitogen-activated protein kinase (MAPK) pathway-dependent and PI3K-AKT-dependent mechanisms in the same patient simultaneously (Shi et al., 2014; Turajlic et al., 2014). Similarly, in one patient with colorectal cancer, through longitudinal tracking of cell free tumor DNA, Diaz et al. (2012) documented four distinct *KRAS* mutations emerging during the acquisition of resistance to panitumumab therapy (targeting EGFR).

Polygenic resistance mechanisms raise clear challenges when considering sequential or combinatorial targeted therapy strategies to forestall acquired resistance events in order to prolong progression free survival times (Burrell and Swanton, 2014). One potential approach in the face of intratumor heterogeneity is to target multiple pathways simultaneously. The emergence of *KRAS* mutant clones in colorectal cancer, which can be detected noninvasively, suggest a strategy for delaying or attenuating drug resistance may involve MEK inhibition as well as anti-EGFR therapy (Misale et al., 2012). Indeed, it has been found that blockade of MEK and EGFR in resistant tumor cells can lead to prolonged ERK inhibition and impaired growth of multiple cell line models (Misale et al., 2014). In the context of *BRAF* mutant melanoma, upfront targeting of both MAPK and PI3K pathways may act to limit the selection of drug-resistance mechanisms and thereby ultimately prolong progression free survival times (Shi et al., 2014), while combined BRAF and MEK inhibition results in improved progression-free survival (Flaherty et al., 2012; Larkin et al., 2014).

However, the health economic and toxicity costs of combinatorial targeted therapeutic strategies attenuating multiple clonal or subclonal driver events, together with the need for evidence of robust and clinically meaningful overall survival endpoints, may limit the utility of these approaches. For this reason, it could be argued that efforts to both understand the biology of cytotoxic response and to improve understanding of DNA damage response pathways should be prioritized as a means to address tumor heterogeneity. Observations that patients having tumors with extreme CIN have improved prognosis compared with those having tumors with intermediate levels of CIN support the notion that manipulating genome instability pathways may provide clinical benefits (Birkbak et al., 2011; Roylance et al., 2011). The potential tractability of this approach is also supported by findings that elevating chromosome missegration rates can be used as a strategy to kill tumor cells or limit tumor development (Janssen et al., 2009; Weaver et al., 2007) and that supplementing cells with nucleosides can reduce the frequency of chromosomal segregation errors (Burrell et al., 2013b).

Immunotherapy

Immunotherapy approaches that do not necessarily depend on the clonality of a single target might overcome some of the challenges of intratumor heterogeneity. Indeed, it has been postulated that the same genome instability processes that drive tumor heterogeneity may concomitantly provide fuel for

personalized immune therapy approaches (Peggs et al., 2007). Specifically, each missense mutation has the potential to give rise to a neoantigen that may be recognized by a patient's own immune system (Rajasagi et al., 2014). Such a scenario has been given support by a recent meta-analysis of five cancer types that found tumors with predicted neoantigens exhibited an improved prognosis compared with tumors without neoantigens (Brown et al., 2014) and emerging evidence that increased benefit from immune checkpoint blockade is observed in NSCLC from smokers, with a higher mutational load, compared with nonsmokers (J.C. Soria et al., 2013, conference). The impact of the neoantigenic repertoire contained within a tumor will also be modulated by the inflammatory environment and whether immune-regulatory checkpoints are permissive for T cell function (Quezada and Peggs, 2013). The relevance of modulating the immune system is underscored by the interest in trials that attempt to remove the immunological brakes that block the induction of anti-tumor responses, for example, through inhibition of CTLA-4 or PDL-1 (Quezada and Peggs, 2013). Clinical studies such as the TRACERx program may permit insights into the relationship between tumor heterogeneity and immune modulation.

New Approaches to Clinical Management

Taking into account the evolutionary dynamics of tumor populations may provide an avenue for therapeutic strategies. For instance, Gatenby's adaptive therapy algorithm suggests focus should be shifted from attempting to eliminate every cancer cell, which may select for resistant untreatable subclones, to controlling cancer growth by understanding and manipulating the selection forces operating within a tumor.

In support of adaptive therapy, in a study of mice injected with ovarian cancer cell lines, it was found that the adaptive therapy algorithm—involving multiple dosages of carboplatin that are lowered when growth is attenuated—resulted in lowered continuous tumor burden compared with a standard treatment involving high dosages of carboplatin (Gatenby et al., 2009). Through adaptive therapy, it is thought tumor cells that are sensitive to chemotherapy are maintained in the tumor population rather than eradicated, preventing competitive release of drug resistant, untreatable subclones.

In practice, however, an adaptive therapeutic strategy would raise some difficulties, including a change of physician emphasis from achieving maximal tumor control to maintaining disease stability. Competitive release of drug resistant subclones following eradication of the drug sensitive clone may be an additional explanation for the lack of overall survival data, despite robust progression free survival times, for many targeted therapeutics (Fojo et al., 2014).

Quantifying Heterogeneity and Identifying Subclonal Mutations

The relationships between diversity within tumors and clinical outcome emphasize the need to develop sensitive clinical tools to quantify intratumor heterogeneity and detect and monitor the dynamics of subclonal events within tumors. Although a plethora of bioinformatics approaches have been proposed for quantifying heterogeneity from both single samples and multiregion samples (for a review, see Ding et al., 2014), deciphering the clonal dominance of a driver event is not necessarily a trivial task. Notably, in ccRCC and NSCLC, variant allele frequencies of known driver events often appear clonally dominant within individual tumor regions but on further tumor sampling are revealed as absent from other tumor regions, giving the illusion of clonal dominance (de Bruin et al., 2014; Gerlinger et al., 2014). In addition, sampling bias due to intratumor heterogeneity is likely to confound the development of companion diagnostics and the implementation of clinically qualified biomarkers (Gulati et al., 2014; Patel et al., 2014; Sottoriva et al., 2013).

It is important to note, too, that current informatics algorithms to define driver genes are likely biased to detect clonally dominant recurrent driver events. When one considers the possibility that each tumor clade may harbor one or more driver events, the number of cancer genes operating in an advanced tumor may rise substantially beyond the current estimates of two to six per tumor (Kandoth et al., 2013). Defining the number of driver events, their subclonal nature, and their potential epistatic relationships will likely require multiregion and longitudinal sequencing of individual tumors and development of cell-free DNA (Diaz et al., 2012; Forshew et al., 2012; Murtaza et al., 2013) and circulating tumor cell technologies (Hodgkinson et al., 2014; Lohr et al., 2014) combined with single-cell sequencing approaches (Hughes et al., 2014; Navin, 2014). Moreover, these techniques may be complemented by imaging that can provide a noninvasive approach for quantifying the extent of heterogeneity within tumors (Aerts et al., 2014). Noninvasive approaches may also be used to track tumor progression during the disease course. In hematological tumors, it has been shown that serial sampling of the same patients is both feasible and informative (Ding et al., 2012; Mullighan et al., 2008; Walter et al., 2012).

An understanding of a tumor's evolutionary history and tumor development also provides evidence highlighting the importance of screening approaches to detect disease early while the tumor bulk and diversity is low. In two NSCLC patients, we found evidence that truncal driver mutations and genome doubling events occurred within a smoking signature context, 20 years prior to clinical detection (de Bruin et al., 2014). These data are consistent with a prolonged tumor latency period after genome doubling and before clinical detection in NSCLC, potentially providing evidence underpinning the efficacy of screening approaches in this disease. On the other hand, it has been shown that the majority of recurrently mutated genes in esophageal adenocarcinoma are also mutated in never-dysplastic Barrett's esophagus, suggesting many potential driver events may be present prior to tumorigenesis and in subclones that will never become cancerous (Weaver et al., 2014). Similarly, pathogenic mutations, implicated in hematological malignancies, have been found in the blood of older individuals, without any evidence of disease (Busque et al., 2012). Conceivably, macroevolutionary events, such as genome doubling and large-scale chromosomal rearrangements may often be required for cells to make the leap from a benign to a malignant phase and therefore may serve as useful markers for clinical risk prediction. Indeed, esophageal adenocarcinomas are characterized by genome doublings and have elevated genetic clonal diversity compared with never-dysplastic Barett's esophagus (Li et al., 2014; Maley et al., 2006).

Tracing the origins of the ''lethal subclone'' may contribute to screening approaches and efforts to stratify risk of recurrence and, in due course, might complement classical histopathological assessment. Haffner et al. (2013) found through longitudinal

assessment of a prostate cancer over a 17-year disease course that the lethal subclone that precipitated the visceral metastatic disease derived from a low Gleason grade region of the primary tumor. The challenge is to move from single case studies to larger longitudinal cohorts in order to study the metastatic process in more detail. Longitudinal studies such as TRACERx combined with postmortem analyses to attempt to map cancer subclones over time (Jamal-Hanjani et al., 2014) will be needed to shed further light on the complex dynamics of tumor evolution.

Conclusions

A primary goal of cancer genomics research has been to provide a catalog of cancer genes and mutational processes that are operative during cancer evolution (Alexandrov et al., 2013; Lawrence et al., 2014). However, a crucial next step in realizing the goals of precision medicine will be to complement this analysis with knowledge of the extent to which key cancer genes and mutational processes are heterogeneous within tumors and their dynamics over time. Complementary to this will be the use of longitudinal cancer genomics data to inform upon the timing of somatic events in relation to the onset of specific forms of genomic instability that might drive tumor diversity.

The study of tumor evolution over space and time has begun to shed light on patterns and processes that dictate the evolution of tumors. It is becoming increasingly apparent that tumors often evolve through a process of branched evolution, and despite substantial heterogeneity, parallel evolution is an emerging theme across malignancies. Studies are beginning to reveal rules governing the temporal acquisition of mutations, with certain driver events found to be predominantly truncal and others occurring later in tumor evolution. These findings, coupled with accumulating evidence that measures of heterogeneity are associated with poor prognosis, mandate the need for further studies exploring the evolutionary history of tumors, their interactions with the host stromal and immune environments, and the rules dictating their progression.

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