

Intratumor Heterogeneity: Evolution through Space and Time

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Abstract

Recent technologic advances have permitted higher resolution and more rapid analysis of individual cancer genomes at the single-nucleotide level. Such advances have shown bewildering intertumor heterogeneity with limited somatic alterations shared between tumors of the same histopathologic subtype. Exacerbating such complexity, increasing evidence of intratumor genetic heterogeneity (ITH) is emerging, both within individual tumor biopsies and spatially separated between biopsies of the same tumor. Sequential analysis of tumors has also revealed evidence that ITH temporally evolves during the disease course. ITH has implications for predictive or prognostic biomarker strategies, where the tumor subclone that may ultimately influence therapeutic outcome may evade detection because of its absence or presence at low frequency at diagnosis or because of its regional separation from the tumor biopsy site. In this review, the implications of "trunk and branch" tumor evolution for drug discovery approaches and emerging evidence that low-frequency somatic events may drive tumor growth through paracrine signaling fostering a tumor ecologic niche are discussed. The concept of an "actionable mutation" is considered within a model of clonal dominance and heterogeneous tumor cell dependencies. Evidence that cancer therapeutics may augment ITH and the need to track the tumor subclonal architecture through treatment are defined as key research areas. Finally, if combination therapeutic approaches to limit the consequences of ITH prove challenging, identification of drivers or suppressors of ITH may provide attractive therapeutic targets to limit tumor evolutionary rates and adaptation. *Cancer Res*; 72(19); 4875–82. ©2012 AACR.

Intratumor Heterogeneity: A New Vision for an Old Problem

Tumor morphologic heterogeneity has long been recognized by pathologists and forms the basis of many tumor grading prognostic classification systems. For example, one component of the breast cancer Scarff–Bloom–Richardson grading system comprises an assessment of nuclear pleomorphism, a characteristic associated with tumor aneuploidy (1). Indeed the term anaplasia, first coined by David Von Hansemann in 1890, refers to nuclear and mitotic atypia and reflects observations of tumor morphologic heterogeneity. Tumor morphologic heterogeneity is often regionally distinct with diversity in tumor cell proliferation, immune infiltration, differentiation status, and necrosis that differ between microscopy fields.

As a result of these common pathologic observations, intratumor genetic heterogeneity (ITH) is considered in tumor immunohistochemical quantitative analyses that often take into account both the intensity of cellular staining

as well as the percentage of tumor cells scoring positive for the immunohistochemical marker. Despite common histopathologic observations of ITH in the clinical setting, our knowledge of the extent of ITH at the genetic or epigenetic level, as well as its underlying causes, has remained relatively rudimentary. Conceptually, the prevalence of ITH and its underlying causes have not been advanced by the routine use of cancer genomics platforms because of the inability of expression microarray or DNA copy number platforms to resolve differences in mRNA expression or DNA copy number between cells of the same tumor.

The advent of tumor deep-sequencing technologies, discussed in this review, has begun to resolve the extent of ITH at the single-nucleotide level. Deep-sequencing technologies are beginning to shed light on the consequences of such heterogeneity for drug discovery and biomarker validation approaches.

An Evolutionary Perspective on Cancer Heterogeneity

The impact of heterogeneity on tumor growth control from an evolutionary perspective has been considered in detail (reviewed in ref. 2). In 1976, Peter Nowell proposed the clonal evolution model of cancer and applied evolutionary models to understand tumor growth and treatment failure and the phenomenon of increased tumor aggressiveness that occurs during the natural history of advanced solid tumors (3). Nowell noted a decade later in a reflection on his work "Tumors arise from a single 'mutated' cell and that biological

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and clinical progression results from subsequent additional alterations, giving rise to more aggressive subpopulations within the original neoplastic clone." He also noted that genetic instability, occurring in tumor cells during disease progression, might enhance this process.

Indeed, subsequent work in the early 1980s from Harris and colleagues, investigating the generation of metastatic subclones from a mouse sarcoma cell line, concluded that the generation of such metastatic clones arose at a higher rate than the generation of stable mutations conferring drug resistance (10–1,000-fold higher rate) and that acquisition of metastatic potential by subclones was in some cases reversible (4), coining the term "dynamic heterogeneity." The parallels with Nowell's concepts of genetic instability and disease progression are intriguing; Harris and colleagues concluded that metastatic variants generated from heterogeneous cell populations might arise through unknown epigenetic mechanisms or other mechanisms generating diversity at a higher rate. Subsequently, the same group found that a highly metastatic melanoma cell line, B16F10, acquired resistance to methotrexate at a higher rate than the B16F1 line with low metastatic potential suggesting a common mechanism responsible for metastatic outgrowth and drug resistance, 2 common phenomena that cooccur in epithelial malignancies (5). The authors concluded that these phenotypes may be unified through one mechanism, mediated by the generation of heterogeneous structural chromosomal gene amplification events from cell to cell that are selected for during drug exposure or metastatic outgrowth.

Developments in the fields of mathematics and evolutionary biology are beginning to shed light on the impact of tumor diversity on evolutionary selection, raising important questions as to how advanced tumors might be more optimally controlled. Gatenby's parallels of tumor metastatic growth with population growth control are prescient: "Two centuries of experience have shown that the vast majority of introduced species are simply too heterogeneous, too dispersed and too adaptive to be eliminated." He proposes that an "adaptive therapy" approach, in which drug-sensitive clones are controlled, but not eliminated, to out-compete drug-resistant but less fit subclones, may prove more tractable in the near term to limit progression of advanced metastatic solid tumors (6, 7).

Intratumor Heterogeneity in Space and Time

In contrast to linear models of tumor evolution with sequentially ordered somatic mutations in driver genes resulting in clonal sweeps of homogeneous tumor cell expansion, more recent evidence suggests that branched evolutionary tumor growth may contribute to ITH both within a primary tumor and between primary and metastatic tumor sites (Fig. 1).

Shah and colleagues investigated a case of advanced invasive lobular carcinoma of the breast and through whole-genome sequencing showed the existence of 19 nonsynonymous mutations present in the metastasis that were not present in the primary tumor diagnosed 9 years before, illustrating the spatial and temporal dynamics of ITH (8). Through elegant FISH-based analyses of acute lymphoblastic leukemia single cells, Anderson and colleagues defined branched evolutionary growth and convergent evolution of recurrent copy number

events occurring in different subclones of the same patient (9). In an extensive analysis of ITH in pancreatic cancer, Yachida and colleagues showed that clonal tumor populations present in the primary, but genetically distinct from the nonmetastatic clones, give rise to metastatic disease in a branched evolutionary pattern, with "progressor mutations" common to metastatic sites and within regionally separated subclones of the primary lesion (10). Campbell and colleagues showed that genome instability occurs early in pancreatic cancer and contributes to ongoing tumor evolution at metastatic sites, distinct from the primary, that may in turn seed tertiary metastases with evidence of convergent evolutionary paths and organ-specific relationships between metastases (11).

Navin and colleagues have provided elegant insight into the depth and challenges of ITH in breast cancer within single tumor biopsies. Using a technique to separate tumor cells based on their DNA content, termed sector ploidy profiling, followed by DNA copy number analysis by comparative genomic hybridization or single-cell sequencing, they showed that a single breast cancer biopsy may contain multiple intermixed karyotypic tumor populations that differ by major structural chromosomal gene amplifications. Such diversity may occur within 1 tumor biopsy or may be regionally separated, related through branched evolutionary growth (12, 13).

Our group analyzed multiple regions of 2 primary clear-cell renal carcinomas and associated metastatic sites (14). Sixty-three percent to 69% of all nonsynonymous somatic mutations identified across multiple biopsies of 2 primary tumors and their associated metastatic sites were not detectable in a single biopsy, suggesting that a single biopsy may underestimate the somatic mutational landscape of a tumor. We found evidence for ITH that was present at genetic, transcriptomic, and functional levels with spatial separation of tumor subclones. Furthermore, between 25% and 50% of nonsynonymous variants identified in 19 single biopsies across 2 tumors were private mutations and evaded detection elsewhere in the tumor despite sequencing to more than 250-fold coverage. Branched evolutionary growth was detected in this analysis with evidence of convergent evolution, with multiple recurrent, yet distinct, inactivating mutations occurring in the same tumor suppressor genes including SETD2, PTEN, and KDM5C in different branches (and regions) of the tumor phylogenetic tree. Therefore, evidence now suggests that despite considerable ITH, it appears there are recurrent targets that are subject to loss of function mutations and convergent evolution, suggesting deterministic tumor dependencies that may be exploitable (9, 14).

Substantiating conclusions of branched tumor growth, Nik-Zainal and colleagues (15, 16) have provided an in-depth whole-genome sequencing analysis of 21 breast cancers. The authors confirm subclonal variation and show that the majority of tumor somatic mutations occur following tumor diversification and branching. Strikingly, all tumors harbored a dominant clone (>50% of cancer cells) that differed by thousands of mutations from other subclones. For example, 1 tumor harbored a dominant clone with 15,600 mutations distinct from subclones within other branches of the phylogenetic tree. By inference, the authors conclude that the

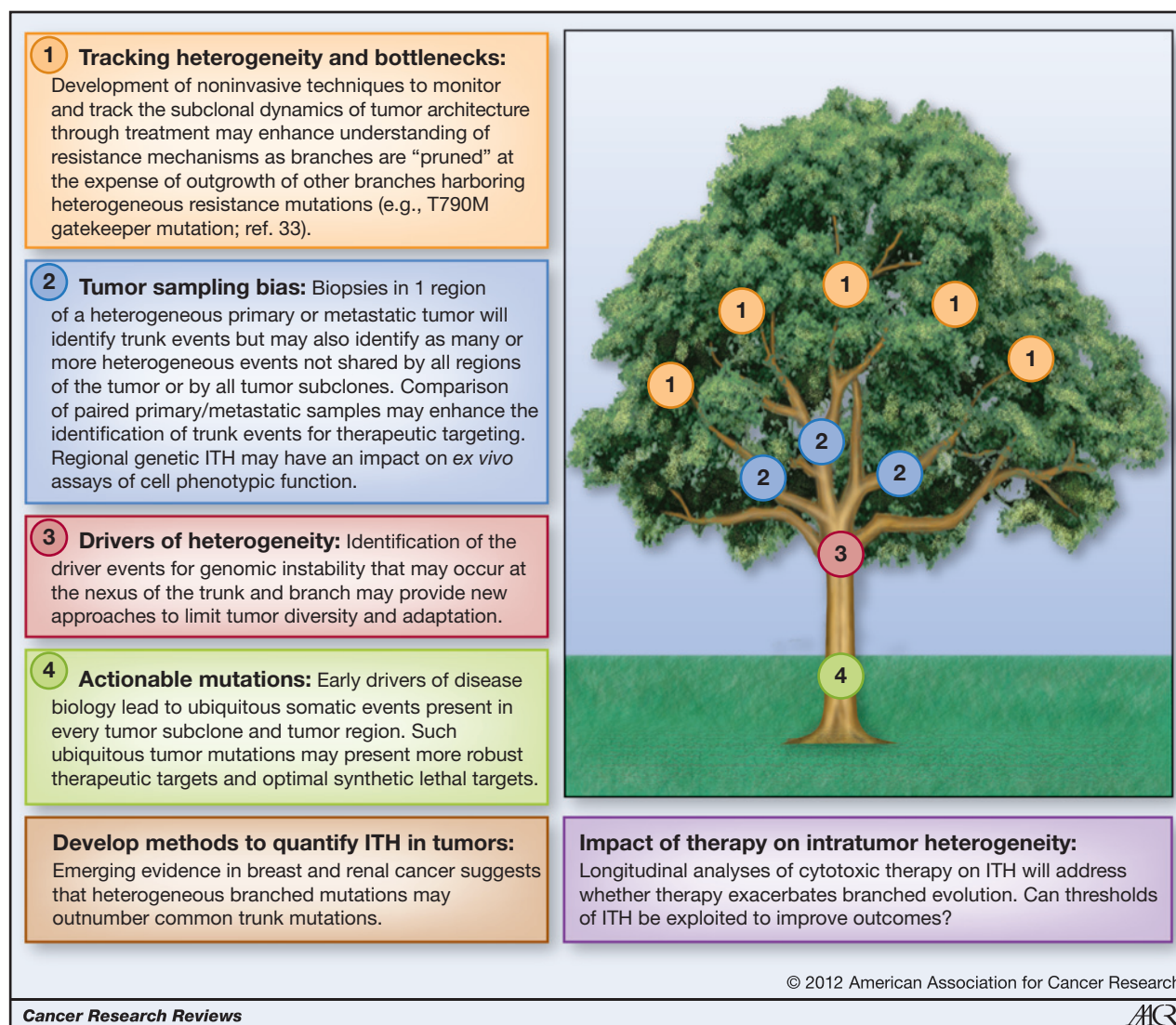


Figure 1. Trunk and branch model representing intratumor heterogeneity. Common or ubiquitous events in the tumor found in every subclone and every tumor region are represented in the trunk of the tree. Diverse, heterogeneous somatic events are represented by the branches and the leaves. Tumor somatic events occurring in the trunk or branches may be driver or passenger events that may be dynamic during tumor evolution and adaptation to therapy. The figure illustrates key research areas and the need to identify how cancer therapeutics might influence intratumor heterogeneity. Adapted from Yap et al. (21).

proliferation and eventual outgrowth of the subclone, precipitating mammographic detection, must have been a rate-limiting event because of the vast number of mutations present in that subclone that differ from the common somatic events present in all tumor cells.

Intratumor heterogeneity and evolutionary bottlenecking

Through the analysis of structural variations and allelic frequencies in a primary basal breast cancer, a xenograft, and a brain metastasis from the same patient, Ding and colleagues showed that the metastasis may derive from a low-frequency subclone within the primary (17). The team noted a wide range of allelic variant frequencies in the primary tumor, indicative of substantial ITH, with less divergent mutational frequencies at the metastatic site,

suggesting a process analogous to evolutionary bottlenecking through subclonal selection during the metastatic process (18). Substantiating the capacity for such heterogeneity to foster metastatic growth, medulloblastoma metastases from the same patient are relatively homogeneous and derive from a low-frequency subclone of the primary tumor (19).

Through our multiregion sequencing analysis of clear-cell renal-cell carcinomas (ccRCC), metastases could be traced back to a distinct region of the primary tumor (14). Consistent with observations from Ding and colleagues, there appeared to be a relative restriction of diversity at metastatic sites (17). Sector ploidy and allelic imbalance analysis of the tumor and metastatic sites suggested that the primary region that spawned the metastasis had become tetraploid with the metastatic sites harboring a chromosomally unstable pattern by

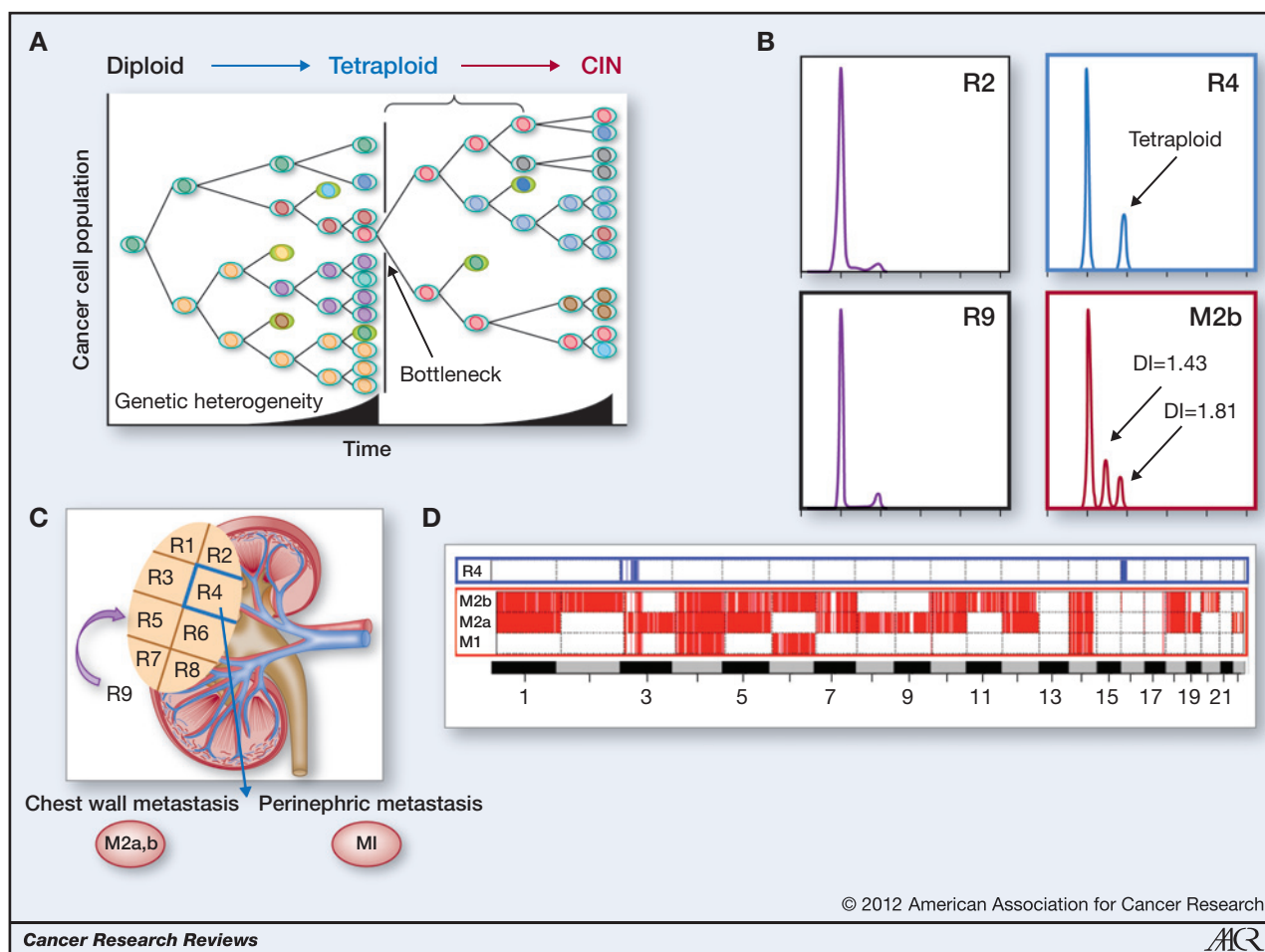


Figure 2. Evolutionary bottlenecking and restriction of diversity between primary and metastases. A, transient restriction of diversity through an evolutionary bottlenecking process, for example, during clonal selection through therapy or during metastatic progression may lead to the requirement for alternative mechanisms to generate ITH. One such mechanism can be generated through structural and whole CIN. Tetraploidy is thought to be a precursor of CIN. B and C, multiregion analysis of a primary renal-cell carcinoma and its metastatic sites revealed that the metastatic lesions were most similar to region 4 of the primary. Ploidy analysis revealed that the primary regions were all diploid with the exception of region 4, which was tetraploid. Metastatic sites were subtetraploid. D, allelic imbalance analysis revealed substantial heterogeneity in chromosome structure between 2 biopsies of the same metastatic site (M2a and M2b). Taken together with ploidy analysis, these data indicate the possible emergence of CIN at metastatic sites. Longitudinal studies will reveal to what extent metastatic sites represent outgrowth of multiple heterogeneous subclones from the primary tumor and genetic events that may be permissive for metastatic outgrowth during the bottlenecking process. Adapted from Gerlinger et al. (14, 18).

allelic imbalance analysis with structural chromosomal complexities that differed between regions of the same metastatic site (Fig. 2). Conceivably, following subclonal selection and the restriction of diversity and bottlenecking, generation of tumor chromosomal instability (CIN) provides a route to rapidly initiate a further expansion in tumor heterogeneity. It is tempting to speculate that this may explain observations of increased CIN at metastatic sites to compensate for the transient restriction of diversity during the selection of a minority tumor subclone (20). The parallels with the work of Harris and colleagues on dynamic heterogeneity are notable as genomic instability driven by heterogeneous structural and numerical chromosomal changes, promoting extensive alterations in gene dosage, may be one such way of creating non-mutational routes to tumor metastases (4, 5). Conceivably, such mechanisms may also be reversible, as postulated in the

dynamic heterogeneity model, as distinct alterations in gene dosage permissive for metastatic outgrowth may be lost in subsequent cell divisions because of spontaneous chromosome reassortments.

The Trunk–Branch Model of Tumor Growth: Delineating Heterogeneous from Ubiquitous Events

Modeling tumor diversity within a tree structure of tumor growth provides a conceptual framework to consider the capacity of tumors to evade cancer therapeutics and the limitations to current biomarker validation strategies (Fig. 1). Early somatic events that drive tumor growth or maintenance, present in early clonal progenitors, are represented within the "trunk" of the tumor (21). For example, somatic events in VHL

(ccRCC) or p53 (triple-negative breast cancer) are often clonally dominant, mapping to the trunk of the tumor evolutionary tree (14, 22). Such trunk somatic aberrations, present at the early stages of tumor development, are likely to be ubiquitous events occurring at all sites of disease. In contrast, later somatic events that occur following branched separation of subclones represent heterogeneous events. Such subclonal heterogeneity may occur within a single biopsy or may be spatially separated between regions of the same tumor or its metastatic sites (14, 21). Intriguingly, evidence is emerging that indicates commonly accepted early drivers of disease biology such as VHL loss (23) and p53, PTEN, or PIK3CA somatic mutations in triple-negative breast cancer (22) may not always be clonally dominant in the tumor population or present in all the tumor cells (p53, PTEN, and PIK3CA in TNBC) or primary and metastatic regions sampled (VHL in renal cancer).

Clinical Implications of Intratumor Heterogeneity

Tumor sampling bias

Tumor sampling bias may arise because of differences in somatic events within the primary tumor, between the primary and metastatic sites, between metastatic sites, or even within single biopsies (Fig. 1). Heterogeneity is also dynamic and evolves over time, as has been observed in elegant FISH studies in cytogenetically high-risk multiple myeloma (24). Dynamic changes in the subclonal architecture of the tumor, where tumor subclones may change and compete with each other for dominance during the disease course and through treatment, create challenges for predictive or prognostic biomarker efforts in which the tumor subclone that defines clinical outcome (e.g., secondary plasma cell leukemia) may not be readily detectable at diagnosis (24).

Therapeutic decision making in oncologic practice is often made with reference to the primary tumor lesion, diagnosed months or years previously, or in cases in which patients present with advanced disease, from one metastatic site. Such approaches are likely to be therapeutically tractable if these tumor somatic events occur in the tumor trunk and are present ubiquitously throughout all tumor subclones and continue to maintain tumor growth and survival at all sites of disease. However, the changing dynamics of tumor subclonal architecture over space and time may result in previously subdominant clones, perhaps either not present or barely detectable in the primary, gaining preeminence. Conceivably, differences in tumor environmental selection pressures at metastatic sites may result in regional variation of tumor subclone evolution as distinct environments select for certain subclones over others, further contributing to ITH (11). Therefore, alterations in the subclonal architecture of the tumor may result in changes in tumor molecular profile that may differ between sites of metastatic disease, distinct from the profile of the primary tumor.

Qualification of clinical biomarkers has been a notoriously difficult process with only 100 of the estimated 150,000 biomarkers qualified and implemented into clinical practice (25). Biomarker discovery approaches combining laboratory analyses of gene function with genetic or transcriptomic analyses of tumor tissue often rely on single tumor biopsies

of the primary or metastatic lesions to prioritize the identification of candidate biomarkers for validation (26). Conceivably, a highly variable genetic and transcriptomic landscape across a primary and metastatic tumor might lead to tumor sampling bias confounding the validation of biomarkers because of spurious associations of heterogeneous tumor genetic events with clinical outcome in the discovery phase of these studies.

Capacity for therapy to augment intratumor heterogeneity

In contrast to mutator phenotypes and microenvironmental factors such as tissue hypoxia and acidosis that may enhance ITH (27), the capacity for cytotoxic therapies to augment ITH remains relatively underexplored. In the study by Keats and colleagues, the enhanced complexity of the relapsed subclone suggested that treatment with DNA damaging agents through myeloma therapy may potentially exacerbate genomic complexity (24). The potential for therapy to augment genome instability and ITH has been observed in relapsed methyl-guanine-DNA methyltransferase-deficient glioblastoma multiforme following alkylator therapy that provides a selection pressure to lose DNA mismatch repair function (28). Similarly, cytotoxic therapy was thought to be responsible for the increase in transversions witnessed in relapsed acute myeloid leukemia following exposure to DNA damaging agents (29). Given the association of ITH with poorer clinical outcome (30), such observations suggest the controversial concept that therapeutics may in some cases contribute to enhanced tumor diversity and adaptation.

Therefore, cancer cytotoxics may contribute to an enhanced tumor evolutionary rate through fostering genetic diversity and, as proposed by Gillies and colleagues, by initiating an adverse tumor microenvironment that enables small phenotypic changes to result in large variations in fitness (27).

Defining actionable mutations within a model of clonal dominance

Tumor deep-sequencing analyses have led to attempts to stratify therapeutics based on the occurrence of "actionable mutations" where a clinician matches a tumor mutation to a cancer drug. Somatic mutational heterogeneity, where distinct mutations may be present in some biopsies but not detectable in others, suggests a more cautionary approach to the clinical implementation of deep sequencing in circumstances where previously undescribed "actionable mutations" might be considered as suitable for clinical trial stratification. Given observations of the spatial separation of somatic mutations, it is conceivable that some actionable mutations, particularly ones with limited prior evidence of relevance in a disease subtype, may not be present within all tumor cells (present at submodal frequencies) or may be regionally separated, and thus represent relatively poor therapeutic targets (Fig. 1). Similarly, synthetic lethal approaches to drug discovery are likely to have optimal efficacy if the genetic dependency of the drug discovery approach occurs early in tumor evolution, present in all tumor subclones, as witnessed by the potent

efficacy of PARP inhibition in tumors of BRCA germline carriers (31).

Perhaps, within the context of early-phase clinical trial development, consideration could be given to the clonal dominance of such "actionable mutations," as sampling multiple sites of disease, to establish the ubiquitous nature of such mutations, is not only traumatic for the patient but also impractical clinically. For example, recent evidence, in glioblastoma and triple-negative breast cancer, indicates that commonly accepted "actionable mutations" in signal transduction pathway regulators such as PTEN or PIK3CA may represent somatic events occurring in the branches of the tumor rather than clonally dominant ubiquitous, trunk mutations (22, 32).

Such considerations should not imply that genetic approaches to treatment stratification from single biopsies are futile. Many of the established biomarkers in oncology derive from, and have been clinically qualified in, single tumor biopsies. Examples include HER2 amplification or overexpression to guide trastuzumab therapy in breast cancer, BRAFV600E mutation to guide BRAF inhibitor treatment in melanoma, and epidermal growth factor receptor (EGFR) activating mutations to guide gefitinib or erlotinib treatment in adenocarcinoma of the lung.

It is likely that many established drivers of disease biology occur early in tumor development and represent ubiquitous events in the "trunk" of the tumor, present at all sites of disease, less subject to tumor sampling bias (21). In the case of renal cancer, such trunk events would be represented by VHL or PBRM1 somatic mutations (14). However, low-frequency somatic events present in some subclones but not others (analogous to the branches of the tumor tree), generating ITH, are likely to contribute to the acquisition of drug resistance driven by Darwinian selection through treatment. Low-frequency gatekeeper mutations in the EGFR tyrosine kinase are associated with inferior progression-free survival on EGFR tyrosine kinase inhibitor therapy (33). Similarly, non-small cell lung carcinoma (NSCLC) exposure to EGFR tyrosine kinase inhibitors can result in the selection of resistant subclones harboring low-frequency mesenchymal epithelial transition factor amplification, present in the tumor before treatment (34).

Such examples suggest that biomarker efforts may have to adapt to the challenge of detecting heterogeneous somatic events present in the tumor at low frequency to predict therapeutic outcome or define combination approaches to limit the acquisition of drug resistance as well as understand the complex phenotypic interplay between heterogeneous cancer subclones. ITH supports a more cautious approach when defining the presence of an actionable mutation for clinical trial stratification to encompass a definition that incorporates clonal or interregional tumor dominance.

Cancer as a complex ecological system dependent on population level heterogeneity

The clonal dominance model to define an actionable event does not necessarily preclude the possibility that tumor growth

can be limited through the targeting of heterogeneous, low-frequency subpopulations. Evidence suggests that minority cancer cell populations may maintain ITH and by inference, targeting such low-frequency cells might have an impact on the bulk tumor population; Low-frequency glioblastoma subclones harboring mutated EGFR maintain ITH through paracrine activation of proliferation of the EGFR-wild-type cancer cell population (35). Considering tumors as ecologic niches with complex functional interdependencies may be necessary to further refine definitions of actionable mutations to attenuate tumor growth. Taking advantage of ITH through enforced competition between tumor subclones forms the basis of elegant models of tumor adaptive therapy (7).

Cancer cell phenotypic heterogeneity and drug sensitivity

ITH is likely to have direct phenotypic consequences on tumor behavior and the acquisition of drug resistance. For example, low-frequency subclones, detectable in the tumor before treatment, harboring resistance mutations in EGFR in NSCLC (33) as well as multiple distinct secondary mutations in c-KIT in different metastases that occur in gastrointestinal stromal tumor following KIT/platelet-derived growth factor receptor (PDGFR) tyrosine kinase inhibitor therapy (36) confer resistance to targeted approaches.

Our group has shown that a heterogeneous mutation near the kinase domain of mTOR promotes resistance to serum deprivation and hyperactivation of the mTOR pathway following everolimus therapy in regions of the primary tumor that harbor the mutation, but not in primary tumor regions with wild-type mTOR. Similarly, extensive ITH in DNA copy number events of "drivers" such as MET, PDGFRA, and EGFR have been shown to occur in a mutually exclusive manner in glioblastoma (37). Adjacent glioblastoma tumor cells display distinct copy number abnormalities in these "targetable" or actionable receptor tyrosine kinase amplification events. Heterogeneous copy number events present in the tumor branches, rather than trunk, suggest that targeting individual branched genetic lesions may prove relatively futile if ITH results in phenotypic heterogeneity in drug sensitivity. Szerlip and colleagues have confirmed this by demonstrating that cell lines grown from the same glioblastoma with heterogeneous PDGFR or EGFR amplification states require both PDGFR and EGFR inhibition for maximal phosphoinositide 3-kinase pathway attenuation and growth inhibition (38). Intriguingly, such heterogeneity is likely to be maintained and selected for by the presence of double-minute chromosomes harboring RTK amplification. Such double-minute chromosomes lack centromeres and are, therefore, unequally segregated during mitosis, resulting in the propagation of ITH. Phenotypic heterogeneity in drug sensitivity profiles that may be spatially separated or present at low frequency in minor subclones of the tumor suggests that profiling cancer cell phenotypes from single biopsies to guide therapeutic decision making from heterogeneous tumors may prove challenging.

In summary, although a proportion of tumors of the same histopathologic subtype may share common drivers, branched events initiating heterogeneity in potential resistance

pathways to targeted therapeutics will likely result in the need to consider individual tumor-specific strategies to extend progression-free intervals. A future in which personalized medicine moves from the current status of patient cohorts defined by single trunk tumor driver events, to the single patient, in which both trunk and branch tumor events are characterized in advance of treatment, and in which no 2 tumors share the same characteristics, may be envisaged. This may have important regulatory, ethical, and health economic implications and raises the need to incorporate an understanding of tumor heterogeneity into clinical trial design. For these reasons, some rightly argue that focusing on the consequences of genetic diversity in terms of common tumor phenotypes, rather than the specific genetic aberrations themselves, may prove more beneficial (27).

Relationships between intratumor heterogeneity and clinical and pathologic parameters

The presence of extensive ITH within primary renal cell carcinomas suggests a rational basis for the improvements in survival outcome associated with palliative surgery to the primary site in patients with oligometastatic disease, through the removal of an evolutionary sink of primary tumor diversity with the capacity to seed further metastases. Longitudinal studies are primed to reveal further insight about the extent to which metastatic sites represent outgrowth of multiple heterogeneous subclones from the primary tumor and genetic events that may be permissive for metastatic outgrowth during the bottlenecking process.

Despite the emerging consequences of ITH on tumor adaptation, little is known about the relationship of ITH with standard tumor histopathologic prognostic parameters, nor how mechanisms generating ITH vary between primary tumors and metastatic recurrences. Developing robust methods to define ITH will be a critical step in this process (Fig. 1). Extensive evidence supports the association of clonal diversity with progression from preinvasive to invasive adenocarcinoma and CIN with poorer disease outcome (30, 39, 40). Prospective analyses of the association of ITH in the primary tumor with risk of early metastatic relapse following adjuvant cytotoxic or radiotherapy seem justified. Such approaches will address whether the degree of ITH might shed light on our ability to cure some primary tumors but not others.

Future Directions

ITH provides a necessary substrate for Darwinian selection during metastatic outgrowth and therapeutic resistance. Subclonal selection and transient bottlenecking that have been

shown to occur during these processes provide both a tool to decipher potential permissive genetic events required during this process and a therapeutic opportunity, if these steps are governed by a restricted set of actionable mutations. Developing minimally invasive approaches to track and monitor tumor subclonal dynamics through the disease course will be an essential step in this process that will also allow the extent to which cytotoxic therapies may exacerbate genomic instability and ITH to be monitored.

Multiregion and ultradeep-sequencing analyses have the potential to shed light on further convergent evolutionary events that tumors must overcome to maintain or continue growth, as evidenced by recurrent distinct SETD2 mutations in ccRCC. Such "predictable" and deterministic tumor dependencies may represent new therapeutic opportunities to mitigate the risk of ITH. Drawing parallels in cancer with examples of convergent evolution in ecology emphasizes the continued need to consider tumor growth within evolutionary and population genetics models (41). Such convergent evolutionary events highlight the requirement to prioritize research on the ecologic tumor niche as the selection force (and in some cases the driver itself of genomic instability) for genetic adaptation, as proposed by Gatenby and colleagues (41).

Continued distinction between trunk and branch events may expand the repertoire of actionable mutations in the tumor trunk, albeit in smaller and smaller patient cohorts. By illuminating common branched events that may predispose to therapeutic failure through subclonal outgrowth, novel combinatorial therapeutic strategies may be envisaged to short-circuit future tumor evolutionary networks and drug resistance mechanisms. ITH may present profound regulatory and practical clinical challenges when considering such drug combinatorial approaches, faced with a restricted number of drugs active against defined actionable mutations compared with the bewildering potential for diversity within individual tumors.

Finally, if such approaches prove intractable, hope may derive from functional studies that illuminate new classes of suppressors and initiators of tumor diversity and immunotherapeutic approaches targeting tumor neoantigenic diversity, which may ultimately lead to therapeutic opportunities to limit tumor adaptation and Darwinian selection.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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