Determinants of phenotypic intratumor heterogeneity: Integrative approach

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Background

Intratumor phenotypic heterogeneity presents a major obstacle toward conceptual understanding of cancer biology, informative clinical diagnostics and successful eradication of cancers with cytotoxic or targeted therapies. Even though intratumor heterogeneity is a subject of intense research focus, our ability to understand the phenomena and address the challenges that it presents is still limited. In part, advances in knowledge are hampered by the fact that diversity of cell phenotypes within a tumor is superimposed over phenotypic differences between different tumors. Furthermore, our ability to understand intratumor heterogeneity is complicated by its multifactorial nature. Historically, phenotypic heterogeneity within tumors has been viewed either under the conceptual framework of genetic heterogeneity, with the assumption that phenotypic differences reflect diversity of tumor cell genotypes, or under the framework of the cancer stem cell (CSC) model, postulating that phenotypic differences are considered to reflect distinct differentiation states. Rather than being mutually exclusive, these frameworks reflect different aspects of the wider phenomenon as neither can fully account for the observed phenotypic diversity. Moreover, since contextual signals are key in determining cellular phenotypes, impacts of alterations and diversity of tumor microenvironments should be considered as well. Finally, impact of increased plasticity stemming from genetic and epigenetic alterations as well as from abnormality of contextual cues needs to be taken into account. This article aims to highlight the multiple inputs that shape up phenotypic heterogeneity as well as outline future directions to improve our advancements in this area.

Discussion

Initiation and progression of cancer is the result of Darwinian-like process of somatic clonal evolution (1, 2). Dysregulated proliferation of tumor cells, genomic instability and stable epigenetic alterations provide for ongoing diversification of heritable phenotypes which serve as a substrate for context-dependent selection forces. Historically, our conceptualization of tumor evolution has been dominated by simplistic model of step-wise clonal expansions (3). However, recent technical advances that enabled high-resolution interrogations of tumor genomes and genetic profiling of single tumor cells have revealed that most primary cancers display complex clonal architecture with co-existence of evolutionary diverged sub-populations that often differ in mutational status of key oncogenes or tumor suppressors (4, 5). Inevitably, this genetic
heterogeneity spills over to phenotypic diversity. Whereas differences in mutational status of key driver mutations have obvious consequences toward tumor cell biology, even ostensibly neutral mutations can have subtle, but functionally important effects on phenotypes due to epistatic interactions and taxation of heat shock protein folding system.

However, phenotypes are not simple functions of genotypes as evidenced by dramatic phenotypic diversity of normal, wild type cells. Therefore, any given aberrant genome is compatible with a range of phenotypic manifestations.

Since malignant clones most likely arise following oncogenic transformation of tissue stem or progenitor cells, they inherit some developmental roadblocks characteristic to the cell of origin, as well as the ability to produce more differentiated phenotypes reflective of lineage differentiation choices available to their wild type counterparts. Parallels between differentiation hierarchies in normal tissues and variations in lineage-associated phenotypes in populations of tumor cells have provided grounds for the establishment of cancer CSC model that views phenotypic variability between tumor cells through the lens of differentiation hierarchy (6).

Popularity of CSC model reflects its substantial explanatory power. Indeed, at least in some cases phenotypic differences that can be mapped to distinct differentiation states are dominant over the impact of altered genomes, as attested by comparison of expression profiling of differentiation marker defined populations between normal and cancerous tissues (7). Still, the CSC paradigm is insufficient to account for full extent of phenotypic diversity.

Differentiation hierarchies are superimposed over genetic differences between distinct subclones within a tumor. On one hand, mutational changes can have substantial phenotypic effects, and, on the other, at least some of the driver mutations can influence differentiation capacity of cells (Fig. 1). To paraphrase a famous analogy (8), differentiation hierarchies in tumors are caricatures of those of normal tissues. Additionally, due to inter tumor heterogeneity each tumor represents a distinct caricature, while intratumor heterogeneity leads to co-existence of multiple caricatures within the same tumor.

Differentiation status of both normal and tumor cells are shaped in response to the contextual signals emanating from microenvironmental niches. Tumor microenvironment possesses some

![Fig. 1. Phenotypic heterogeneity in tumor cell populations. Letters denote genetically distinct sub-clones, while shapes denote phenotypic states that can be mapped to differentiation hierarchies. Phenotypic heterogeneity in populations of tumor cells reflects influences of both genetic alterations and available differentiation states. Phenotypic diversity is further augmented by abnormal features of tumor microenvironment as well as transformation-related changes as explained in the text.](image)
unique features that are not seen in normal tissues, such as acidification, hypoxia and elevated interstitial pressures. Moreover, orderly spatial organization of niches observed in normal tissues is also substantially altered and "messed up" in tumors, leading to substantial variability in contextual signals. For example, tumor specific abnormalities in vascularization lead to substantial diversity of availability of nutrients and oxygen over space and time (9). Therefore, both identity and diversity of contextual signals are altered in tumors compared to normal tissues.

In addition to abnormal signals emanating from the tumor microenvironment, phenotypic states of tumor cells are likely to be affected by their internal ability to respond to these cues. For example, highly recurrent driver mutations (such as mutation of RAS or inactivation of PTEN) lead to activation of signaling pathways that become independent of receptor-mediated stimulation. Furthermore, epigenomes of tumor cells are characterized by profound perturbations in DNA methylation and histone modifications, affecting epigenetic landscapes (potential phenotypic states available to the cells). Moreover, epigenetic alterations, saturation of cellular stress responses to misfolded proteins and increase in gene expression noise are likely to lead to reduced stability of a given phenotypic state (10). Indeed, cancer cells appear to possess increased plasticity compared to their normal counterparts, as attested by elevated capacity for de-differentiation (11).

Obviously, meaningful experimental studies require reductionist approaches with research questions being formulated within the confines of a given framework. However, in order to achieve adequate conceptual understanding of intratumor heterogeneity with the purpose to improve diagnostics and therapeutic outcomes will require consideration of all the major determinants of phenotypic heterogeneity (Fig. 2).

Fig. 2. Determinants of cancer cell phenotypes. Phenotypes of individual cancer cells integrate inputs from genetic mutations, differentiation options (combination of normal differentiation options and cancer-specific epigenetic alterations), contextual signals (signals from secreted factors, extracellular matrix and cell-cell interactions), and stochastic fluctuations in gene expression. These inputs are not entirely independent as denoted by curved arrows. Genetic aberrations and contextual signals affect differentiation options; chromosomal instability and accumulated mutations are likely to increase stochasticity in gene expression. Abnormal nature and diversity of contextual signals, together with increased expression stochasticity are expected to increase phenotypic plasticity.
Future Directions

Despite substantial experimental attention, our knowledge on phenotypic heterogeneity in primary tumors remains limited. This needs to be changed if we are to achieve progress in clinical diagnostics and therapy. Recent technical advances that enabled deep sequencing of tumor genomes and bioinformatical deconvolution of the sequencing data gave us the ability to decipher genetic clonal architecture of populations of tumor cells. Ideally, we would like to gain similar information on phenotypic architectures and interrogate how this phenotypic architecture is altered in response to anticancer therapies. Unfortunately, deconvolution of bulk expression profiling data is not feasible and our advances in knowledge have to be based on single cell analysis. So far, most of single cell based studies have been based on the CSC paradigm, with interrogated differences limited by use of a priori selected small number of differentiation markers. Fortunately, technical advances in RNA sequencing enabled expression profiling of single cells (although high cost and poor signal to noise still pose formidable challenges), allowing for unbiased and high-resolution identification of distinct phenotypic states within cell populations. Whereas high cost and poor signal to noise ratios limit the wide use of single cell expression studies, these studies should be able to provide us with phenotypic markers allowing to define phenotypic architectures using more scalable techniques, such as mass cytometry (12). Furthermore, phenotypic markers can be employed in the analysis of tissue sections with mass spectrometry (13) or fluorescence microscopy based techniques, enabling the analysis of small diagnostic specimens, as well as gaining additional knowledge, such as dependence of specific phenotypic states on factors such as oxygenation, proximity to fibroblast, etc.

Gaining unbiased knowledge on phenotypic heterogeneity of tumor cell populations could enable us to ask which aspects of phenotypic architecture can predict therapeutic response, potentially enabling the developing of new diagnostic markers that would be obscured with population-based analyses. Furthermore, knowledge about spatial phenotypic heterogeneity could be used to direct the development of more effective tumor sampling approaches or at least to achieve clearer understanding of the topology-related limitation of current techniques.

Common response to therapy involves initial tumor shrinkage with survival of cells harboring partially drug-resistant phenotypes, followed by recurrence typically driven by outgrowth of drug-insensitive sub-clones harboring some resistance-conferring genetic changes. We expect that selective pressures imposed by cancer treatments, differences in drug sensitivity among different phenotypic states, and adaptive phenotypic changes induced by the treatment, should reduce phenotypic heterogeneity. Thus, identification of phenotypic states linked to drug resistance and uncovering their unique vulnerabilities could substantially improve clinical response, and, ideally, achieve complete eradication of tumors.

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