The existence of intratumor phenotypic heterogeneity was described a few decades ago, but the biological impact of this clonal diversity remains unknown. In 1976, Peter Nowell stated in *Science* that tumors arise from a single cell that has undergone several expansion rounds and whose descendants eventually acquire genetic aberrations. He also posited that additional mutational events would drive the behavior of the tumor and determine how the tumor will evolve (1). Nowell proposed the concept that tumor progression is linked to a genetic evolution that follows a Darwinian pattern, thus bringing the notion of tumor evolution to bear on this heterogeneity. Since that time, numerous studies have reported the presence of subpopulations within tumors, and it has been proposed that this clonal diversity could affect tumor evolution by (a) providing the diversity on which selection can work and (b) modulating progression and resistance through biological interactions between the clones (2).

The New England Journal of Medicine recently published a report by Gerlinger et al. entitled “Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing” (3), which evoked the concept of intratumor heterogeneity playing a role in cancer progression and metastasis and in complicating molecularly driven treatment strategies. The authors investigated primary renal carcinomas and their associated metastases via multiregion whole-exome sequencing, chromosome aberration analysis, and DNA ploidy profiling of spatially separated parts of each sample. Immunohistochemical and gene expression analyses were also performed. Their data showed that 63% to 69% of all somatic mutations were not detectable across every tumor region and that a phylogenetic reconstruction of the tumors revealed an evolutionary tumor growth pattern. Additionally, the authors observed different gene expression signatures and divergent allelic-imbalance profiles within some of the tumors. The authors concluded that a single tumor biopsy sample might reveal only a fraction of the genetic aberrations present and therefore might not represent the diversity present in the entire tumor. Furthermore, they showed that reconstructing the tumor clonal architectures by localizing aberrations in the root of the phylogenetic tree might bring substantial insights that could contribute to improving therapeutic approaches, if these “root” events can be targeted.

Although the magnitude of intratumor heterogeneity and the mutational status at different cancer stages are unclear, several studies have demonstrated that the clinical implications can be huge. One study has shown a strong correlation between the degree of clonal diversity and the probability of invasive progression of Barrett esophagus to esophageal lesions, thus correlating heterogeneity with the development of malignancy (reviewed in (2)). Other studies have shown that mutational status can predict resistance to treatment by preventing the binding of a drug to its target. One of the best examples is the BCR-ABL fusion protein in chronic myeloid leukemia. Its presence is a predictor of a patient’s response to imatinib mesylate, but some mutant forms of the BCR-ABL protein have been implicated in the relapse of chronic myeloid leukemia. This pattern has been found with other tumor types, including melanoma and gastrointestinal stromal sarcomas (also reviewed in (2)).

With the accumulating evidence that intratumor heterogeneity seems to be closely related to tumor progression and the development of resistance to therapy, one of the most challenging aspects of studying this heterogeneity is to determine how best to account for heterogeneity when planning sample acquisition for molecular analysis and clinical decision-making. A decision about a patient’s treatment might require consideration of not only the dominant clone but also any minor clones that might present clinically relevant aberrations. As Gerlinger et al. (3) and others have indicated, a single biopsy may not reflect the composition of the entire tumor, owing to the clonal heterogeneity. One option is to collect samples from spatially separated parts of a tumor for analysis. Although this approach might be feasible for surgical samples, it is more...
challenging when primary tumors or their metastases are sampled percutaneously. Clearly, this strategy presents many obstacles, including the resulting morbidity and the high costs of multiple biopsies, among others. Besides sample size issues, another challenge is that most clinical samples are fixed in formalin and then preserved in paraffin, a process that often decreases DNA and RNA integrity and quality. Fortunately, advances are rapidly being made in the use of formalin-fixed paraffin-embedded specimens for next-generation sequencing. An alternative approach is to treat the patient according to the information derived from the available samples and then rebiopsy the patient later if the tumor progresses. That approach may prove more practical in cases in which large tumor samples are not readily available. Alternatively, circulating free DNA or circulating tumor cells may reflect not only the heterogeneity in the primary tumor but also that in distant metastatic sites.

Many researchers continue to search for the best tools and strategies to identify and correlate tumor heterogeneity with prognosis and treatment. Although the number and size of tumor samples from a patient are frequently limited, new technologies might provide a solution for evaluating intratumor heterogeneity when the available samples are inadequate. With the advent of next-generation sequencing and the recent reductions in its costs, the detection of mutations at very low frequency (i.e., those represented by small clones in the tumor population) seems to be possible. In this context, one of the main strategies would be to sequence samples at a very high depth. In their *New England Journal of Medicine* report (3), Gerlinger et al. performed whole-exome sequencing to a depth of >30-fold (maximum, 117-fold). Although this depth of sequencing is sufficient for confidently calling major clones, it would be challenging for identifying minor clones. Achieving a substantially greater depth is possible with targeted exome sequencing, whereas achieving that goal for whole-exome sequencing (let alone for the entire genome) would be very challenging, owing to both the prohibitive cost and the magnitude of the data analysis. Additionally, sequencing to great depth could identify rare cells from other parts of the tumor that have migrated to the site of biopsy or, alternatively, reseeded the primary tumor from distant sites. It is important to mention, however, that high-depth targeted sequencing has the challenge of false-positive and false-negative calls for rare clones. The development and use of new algorithms that can accurately call mutations by distinguishing real aberrations from false positives and negatives is a key component for identifying clonal subsets with targeted gene sequencing. Additional research that uses the targeted approach is necessary to determine whether it can adequately identify the minor clones that may be driving tumorigenesis, metastasis, or resistance to therapy.

An elegant method for inferring tumor evolution and addressing sample size issues with single-cell sequencing has been described by Navin et al. (4). The use of flow-sorted nuclei and whole-genome amplification followed by next-generation sequencing allowed the authors to identify and quantify genomic copy number within an individual nucleus at a robustly high resolution. The findings from this study are consistent with those obtained with pooled DNA from a tumor, demonstrating that copy numbers in primary and metastatic sites are similar and suggesting that metastatic cells emerge from an expansion of the primary population late in tumor development. Moreover, the data indicate that tumors grow by punctuated clonal expansion. Such growth reflects the sudden emergence of a tumor cell with a markedly increased growth capacity. This approach is still in its infancy, however, and much work is needed to determine the number of cells necessary to characterize the clonal diversity of a tumor. Additionally, this approach requires substantial bioinformatics prowess not typically available in most clinical settings.

As we have alluded to above, it is important that a comprehensive analysis of intratumor heterogeneity also rely on appropriate analysis tools. Merlo et al. (5) analyzed >200 Barrett esophagus cases with all major methods for measuring diversity, including genetic-divergence and entropy-based measures, to determine which diversity measurement methods are correlated with the risk of progression to esophageal adenocarcinoma. Together, the use of new methodologies (e.g., high-depth sequencing, single-cell sequencing), combined with the appropriate analysis tools, to detect and interpret intratumor heterogeneity could substantially improve clinical-management decisions and patient outcomes.

The report by Gerlinger et al. elegantly demonstrates the problems we face with intratumor heterogeneity. Going forward requires equally careful studies to determine whether targeted exome sequencing to very high depth or perhaps multiple single-cell sequencing runs can capture the heterogeneity in a tumor and the complexities that each patient faces. Such insights will lead to a more detailed understanding of the treatment options. Further developments in analytical tools would aid in reducing the time required to analyze the large data sets acquired with these approaches. Although the heterogeneity demonstrated in this report may seem daunting, it illustrates perfectly what many researchers have believed and lays the foundation for discovering a solution that improves decision-making for cancer treatment and the outcomes of such treatment.
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