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Cellular Heterogeneity and Molecular Evolution in Cancer

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Abstract

Intratumor heterogeneity represents a major obstacle to effective cancer treatment and personalized medicine. However, investigators are now elucidating intratumor heterogeneity at the single-cell level due to improvements in technologies. Better understanding of the composition of tumors, and monitoring changes in cell populations during disease progression and treatment, will improve cancer diagnosis and therapeutic design. Measurements of intratumor heterogeneity may also be used as biomarkers to predict the risk of progression and therapeutic resistance. We summarize important considerations related to intratumor heterogeneity during tumor evolution. We also discuss experimental approaches that are commonly used to infer intratumor heterogeneity and describe how these methodologies can be translated into clinical practice.

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Intratumor heterogeneity: variability in cell phenotypes within a tumor caused by genetic or nongenetic sources of variability

Biomarker: "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (154, p. 91)

1. INTRODUCTION

Intratumor heterogeneity for cellular phenotypes has been recognized for a long time. Virchow, considered the father of modern pathology, observed pleomorphism of cancer cells within tumors in the nineteenth century (reviewed in Reference 1). Studies performed as early as the 1950s investigated functional and genetic heterogeneity within spontaneous tumors in animals by assessing cytogenetic profiles and tumorigenicity at the single-cell level (2). Elegant studies by Heppner, Fidler, and their colleagues (3–5) in the 1970s demonstrated the existence of distinct subpopulations of cancer cells within tumors, which differed in terms of tumorigenicity, resistance to treatment, and ability to metastasize. The advent of molecular biology and the development of monoclonal antibodies to cell-surface markers opened up new possibilities for in-depth analyses of tumors, including the ability to assess genetic variability among individual cancer cells (6). The clinical implications of heterogeneity in cellular phenotypes within and between tumors were also recognized long ago. In particular, investigators recognized that variability in the expression of biomarkers among distinct tumor subtypes can aid treatment decisions but that variability within tumors poses a challenge in the management of cancer patients (7).

The increasing focus on the tumor stem cell hypothesis in recent years has stimulated interest in intratumor phenotypic heterogeneity, which can now be studied at the single-cell level thanks to new technologies that have led to the rapid accrual of knowledge in this area (8–10). Our new knowledge includes important mechanistic and conceptual insights into the nonheritable heterogeneity of cellular phenotypes; these insights have arisen from recent research focused on the stochastic nature of biochemical processes within cells and their impact on epigenetic landscapes (11). Intratumor heterogeneity is evolving beyond a simple tumor trait toward prognostic and predictive biomarkers for assessing the risk of tumor progression and therapeutic resistance, respectively (12).

Initiation and progression of tumors are associated with the development of obligate characteristics, defined as hallmarks of cancer, that distinguish tumor cells from their normal counterparts. These traits include sustained proliferative signals, ability to evade growth suppressors, immune evasion, promotion of inflammation, replicative immortality, increased motility, metastatic ability, angiogenic potential, increased genomic instability, resistance to cell death, and altered cellular metabolism (13). However, whereas hallmarks of cancers are properties of populations of tumor cells (and the intermingled stroma), individual cells within a given tumor often display variability in these traits. Phenotypic features that display substantial cell-to-cell intratumor variability include activation of signaling pathways, evasion of antitumor immunity, induction of senescence, production of secreted factors, migration, metastasis, angiogenic capacity, genetic makeup, response to anticancer agents, and activation of metabolic pathways (**Figure 1**). Furthermore, intratumor heterogeneity applies not only to tumor cells but also to components of the microenvironment (14, 15).

In the clinical setting, intratumor heterogeneity poses a challenge for personalized cancer diagnosis and treatment selection. For instance, cancer diagnosis is performed on biopsies of a small region of a tumor, which may not necessarily provide representative biological information for the tumor as a whole. Therefore, intratumor heterogeneity for the expression of some proteins could significantly affect the effectiveness of targeted therapies, wherein the biomarker used for diagnosis is often the target for treatment. In addition, intratumor variations of biomarkers could affect the success of biomarker-driven clinical trials if the biomarker used to predict response and to classify patients into subgroups displays spatial variability.

Intratumor heterogeneity measurements could also be used as risk-stratification markers. To apply intratumor heterogeneity measures in clinical practice, investigators will need to answer many of the following questions: Which heterogeneous tumor trait should be measured,

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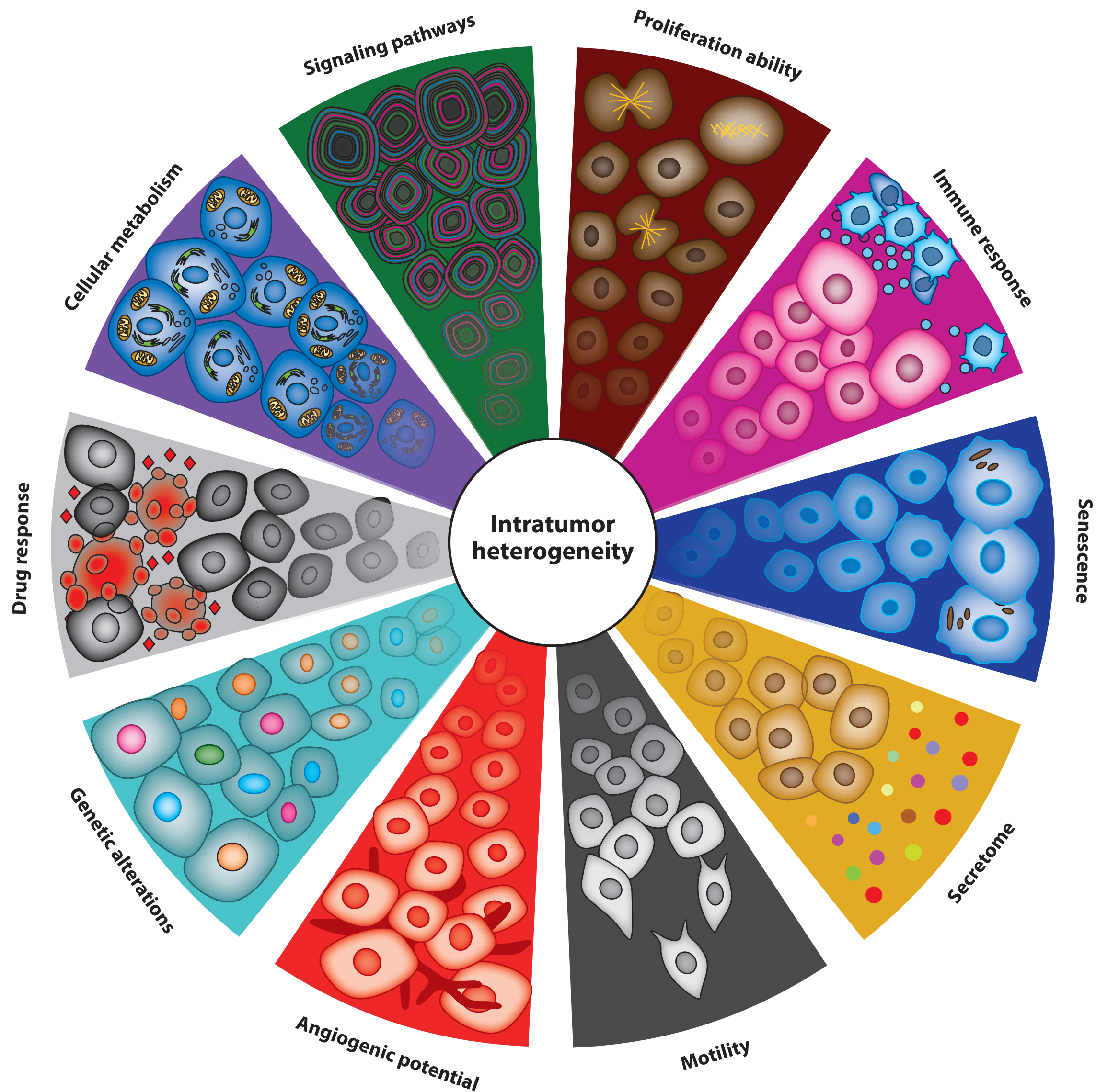

Figure 1

Heterogeneity and the hallmarks of cancer, which constitute approximately 10 distinct biological properties that all tumors share. Tumors exhibit varying degrees of intratumor heterogeneity in each of these properties. This heterogeneity may be regional (due to the presence of subclones that dominate different parts of the tumor or primary tumors and metastases) or mosaiform, which refers to cells with different properties that are closely intermingled within a tumor. This diversity in cellular properties ultimately affects therapeutic responses and disease outcomes.

and does heterogeneity vary according to different types of measurements? Does heterogeneity influence the predictive value of all biomarkers or only some of them? Can genetic heterogeneity alone be used for risk stratification and to guide treatment decisions? If so, what is the best way to define genetic heterogeneity: by assessing either single cells or bulk tumors by use of high-coverage whole-genome or whole-exome sequencing? Addressing these

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Genetic diversity: presence of genetically distinct cells within a population

Clone: group of cells of the same genotype derived from a common ancestor; here, refers to tumor cells that have the same tumor-driving mutation

CIN: chromosomal instability

Driver mutation: mutation that provides selective advantage to a clone by increasing either its survival or its reproductive success

Fitness: ability to survive and reproduce in a particular environment

questions will provide molecular pathologists with valuable tools to design personalized therapies, whose objectives are to deliver effective treatments that are customized for each individual and to spare patients who are not likely to respond from unnecessary toxicity (16–19).

Here, we provide an overview of our current knowledge of intratumor heterogeneity, the methodologies used to measure it, and its clinical implications. Moreover, we emphasize the importance of translating these concepts and approaches into clinical practice; the ultimate goal is to achieve a truly personalized medicine for cancer treatment.

2. INTRATUMOR HETEROGENEITY AND TUMOR EVOLUTION

2.1. Sources of Intratumor Heterogeneity

Phenotypic heterogeneity within populations of tumor cells is a complex phenomenon that integrates genetic diversity and nongenetic sources of heterogeneity.

2.1.1. Genetic heterogeneity. The discovery of recurrent genetic mutations that drive the development of spontaneous tumors, as well as demonstrations that normal cells can be transformed into tumorigenic ones by expression of mutant genes, led to the development of gene-centric views of cancer, which have dominated cancer biology for several decades. In this perspective, phenotypic differences between cells within a tumor are thought to reflect genetic differences between them (20). How does genetic diversity in a population of tumor cells arise? In most cases, all of the cells within a tumor are thought to be derived from a single initiating transformed cell, and are therefore clonal. However, the process of tumor development allows multiple routes of genetic diversification. First, researchers believe that in most cases, the progression of the initiated clone toward malignancy requires that the clone overcome telomere crisis, a period of chromosomal instability (CIN) triggered by critically shortened telomeres that is associated with multiple cycles of chromosomal breakage bridging. This process leads to multiple chromosomal translocations and aneuploidy (21, 22) and sometimes to dramatic whole-genome chromosomal reshuffling (23). Therefore, whereas most premalignant cells are eliminated through the induction of apoptosis, mitotic catastrophe, or senescence, cells that manage to escape from telomeric crisis do so through a process that generates a substantial mutational load and genetic diversity. Second, most cancers display a marked increase in the rates at which they acquire genetic mutations, mostly in the form of CIN (24); increased genetic instability is considered one of the so-called hallmarks of cancer (25, 26). Third, although elevation of mutation rates in cancer is commonly accepted, driver mutations required for the causation of advanced malignancy may be acquired through random mutagenesis, even without increased rates in DNA mutations (27). This hypothesis highlights the importance of multiple rounds of cell division, which are required for the formation of macroscopic tumors, in the diversification of genomes. Importantly, the number of cell divisions required to form a clinically diagnosed tumor may be substantially larger than that inferred from the tumor mass, given that the proliferation of tumor cells is frequently counterbalanced by cell death and by cells switching into nonproliferative states. Indeed, large tumor size is usually associated with higher genotypic diversity (28).

In addition to genetic diversification that arises from errors in DNA replications, another level of genetic complexity can be attributed to clonal diversity: the coexistence of genetically diverged clonal subpopulations. Cancers are thought to result from selection between genetically distinct subclones that arise from somatic mutations (29). This process is essentially Darwinian: Genetically and epigenetically diversified heritable phenotypes are tested by selection, which causes the preferential outgrowth of clones with higher-than-average fitness. Similarly to evolutionary processes in natural populations, tumor evolution is

characterized by complex dynamics that produces unique and unpredictable patterns of clonal architecture (30).

Whereas not all of the genetic mutations have phenotypic manifestations, and only a fraction of those are likely to have biological consequences, at least some genetic diversity is likely to influence clinically and biologically important traits. Indeed, as we discuss below, in many cases therapeutic failure has been attributed to the outgrowth of genetically distinct clones that were present before the onset of therapy. Furthermore, because many cancers have hundreds of thousands of genetic mutations, phenotypically silent mutations probably display phenotypic manifestations due to genetic interactions with other silent mutations (31). Finally, many tumors display activation of heat shock responses (32), and because heat shock proteins have been implicated in the so-called canalization of phenotypes (33), saturation of heat shock responses can cause mutations that would be silent under normal circumstances to produce phenotypes.

2.1.2. Nongenetic heterogeneity: epigenetic mutations. Heritable changes in phenotypes of tumor cells are not limited to those arising from differences in DNA sequences. Development of cancers is associated with epigenetic abnormalities that affect multiple aspects of cellular biology and differentiation status (34). Some of these changes can be functionally similar to genetic mutations and, therefore, are frequently referred to as epimutations (35). For example, inactivation of the *CDKN2A* (*p16^INK4A^*) tumor-suppressor gene is one of the most frequent recurrent events in diverse cancer types. This inactivation can arise from either genetic mutations (either point mutations or homozygous deletions) (36) or epigenetic inactivation due to promoter hypermethylation (37). However, the effect of epigenetic silencing is not strictly equivalent to that of genetic mutation. Apart from the potential reversibility of the epigenetic changes, epigenetic silencing frequently affects multiple loci, which leads to phenotypic consequences that are more complex than a specific mutation in a single gene (38). For example, the above-mentioned epigenetic silencing of the *p16^INK4A^* promoter has been linked to the epigenetic silencing of *HOXA9*, a homeobox gene involved in the regulation of breast epithelial cell differentiation (39). Still, given that epigenetic inactivation can be heritable and have a fitness benefit, it can serve as a substrate for clonal evolution. Interestingly, genome-wide hypomethylation, a characteristic of most human cancers, has been associated with increased rates of CIN (40). Another interesting aspect of epigenetic changes is that some can be reversed after multiple rounds of population doublings. Therefore, phenotypic traits that arise from these changes are not fixed; rather, they constitute a "gray zone" of tumor evolution. Although this aspect of cancer biology has received relatively little conceptual and experimental attention, these semiheritable traits may "lubricate the machinery of natural selection" (41, p. 340).

AML: acute myeloid leukemia

2.1.3. Nongenetic heterogeneity: differentiation hierarchies. The dominance of purely gene-centric views on determinants of cancer cell phenotypes ended in part because of the emergence of the cancer stem cell paradigm. Although the idea that phenotypic distinctions within tumors are a reflection of differentiation hierarchies similar to those found in normal tissues has been circulating for a long time (for an excellent historical perspective, see Reference 42), the idea attracted the attention of the wide research community following a seminal discovery and publication by Bonnet & Dick (43). Specifically, phenotypic markers ($CD34^{+}CD38^{-}$) that distinguish stem cell–enriched populations in the normal hematopoietic system identified a subpopulation of cancer cells in acute myeloid leukemia (AML) that were uniquely capable of propagating the disease and recreating phenotypic diversity in immune-compromised mouse recipients (43). This study was followed by other reports that identified phenotypically distinct subpopulations enriched in cells with

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tumor-initiating capabilities in many other cancer types, including not only hematopoietic but also solid tumors (reviewed in References 42 and 44). These observations helped initiate a burgeoning field of cancer stem cell research.

Drawing parallels between differentiation hierarchies in normal tissues and tumors has clear limitations, and a popular metaphor describes differentiation hierarchies in tumors as "caricatures of corresponding normal tissues" (45, p. 267). In an extension of this metaphor, because every spontaneous tumor has a unique evolutionary trajectory, each tumor must have a unique caricature. Indeed, the $CD34^{+}CD38^{-}$ immunophenotype, which was originally used to identify cancer stem cells in AML, varies among individual tumors (46), and in some cases most tumor-initiating cells are found in a fraction that displays markers of differentiated cells (46). Furthermore, cancers display a large spectrum of epigenetic abnormalities, and the number of distinct epigenetic states (and corresponding phenotypes) probably greatly exceeds the number of epigenetically determined states of differentiation found in normal tissues. Therefore, whereas the cancer stem cell paradigm is clearly useful for understanding tumor biology and in clinical applications, the concept of differentiation hierarchy within tumors does not provide a comprehensive explanation of nongenetic sources of intratumor phenotypic heterogeneity.

2.1.4. Nongenetic heterogeneity: stochastic mechanisms. Single-cell analysis of genetically identical cells grown in tissue culture inevitably reveals cell-to-cell variability in virtually all discernible phenotypic traits (47). This variability arises from the stochastic nature of biochemical processes within cells. The best-studied process is variable gene expression, which is thought to arise from the inherently stochastic nature of transcription (most genes are represented by two alleles, which inevitably leads to some fluctuations in expression) and the "burst-like" way in which most eukaryotic genes are transcribed. The mechanism of such transcription is not well understood, but it probably involves changes in chromatin states (48).

The most prominent implication of stochastic fluctuations in cellular phenotypes is probably the differential sensitivity of cells within populations to ligand-based (49) or cytotoxic (50) therapies. This differential therapeutic sensitivity, which arises from noise-driven cell-to-cell differences in expression levels of apoptotic machinery elements, may underlie the "fraction cell kill" concept, namely a hypothesis, supported by substantial experimental evidence, that a chemotherapeutic regimen kills a fraction of cells within a population irrespective of the population's size (51). Importantly, stochasticity in gene expression may be responsible for transitioning between distinct epigenetic states associated with clinically important phenotypes. For example, in bacteria, stochastic gene expression is thought to be responsible for a phenomenon termed persistence, a phenotypic state that is associated with reduced proliferation rates but increased resistance to antibiotics (52). The subpopulation of persistent cells spontaneously and constantly arises in clonal and genetically identical bacteria grown in homogeneous conditions. A similar mechanism might be responsible for the reported spontaneous development of distinct epigenetic states associated with drug resistance in cancer cells (53).

The stochasticity of gene expression can also mediate transitions between distinct differentiation states. Clonal populations of murine hematopoietic progenitor cells spontaneously generate outlier subpopulations that can be differentiated by either very low or very high levels of expression of Sca-1. These subpopulations have distinct transcriptional profiles that underlie a differential bias toward differentiation into erythroid and myeloid lineages (54). In this case, global gene-expression patterns associated with erythroid and myeloid differentiation may represent metastable attractors in gene-expression networks, in which individual cells can transition between distinct attractor states owing to stochastic noise in gene expression. Similar mechanisms may be

responsible for subpopulations with functionally distinct properties in clonal populations of tumor cell lines cultured under homogeneous conditions (55, 56).

2.2. Clonal Architecture of Tumors

According to a common, textbook model of clonal succession, somatic evolution in cancer proceeds as a stepwise series of clonal expansions. This process is triggered by the acquisition of driver mutations that confer strong fitness gain and cause clonal homogenization, as the more advanced clone outcompetes its less fit parental and sister clones (57). In this scenario, most tumor cells should be genetically identical, even though they may carry large numbers of mutations. In many cases, queries of intratumor genetic heterogeneity reveal genetic homogeneity, which is consistent with the model of clonal succession (58). Although the detection of genetic heterogeneity might be hampered by the limited resolution of many techniques that investigate clonal architecture, some tumors display apparent genetic homogeneity even at the single-cell level and at genome-wide resolution (8). However, determination of intratumor clonal composition is complicated both by sampling issues and by limits in resolution of the analysis (58). Nevertheless, a growing body of experimental evidence supports the existence of complex subclonal architecture in human cancers (8, 58, 59). Furthermore, recent advances in DNA-sequencing technologies allow investigators both to perform unbiased analyses of tumor genomes at high resolution and to make inferences about the clonal composition of tumors (60). Indeed, recent experimental reports utilizing massively parallel sequencing technologies have revealed complex clonal architectures both in primary tumors and between primary tumors and metastatic sites (61–63). Interestingly, complex clonal composition was recently described in acute lymphoblastic leukemia (ALL) (64, 65), a hematopoietic malignancy with relatively few genetic aberrations and recurrent driver mutations. This observation suggests that complex evolutionary dynamics and the resulting complex clonal architecture are probably common features of all cancers.

Whereas ongoing clonal diversification is an inevitable consequence of evolutionary dynamics, several mechanisms, including spatial constraints and differences between the selective pressures of different localities within a given tumor, can further contribute to the maintenance of clonally heterogeneous tumors (58). Importantly, given that some epigenetic alterations behave as heritable traits, clonal architecture is not limited to genetically distinct cells; it also includes subclones with distinct phenotypic features that arise from heritable epigenetic differences.

ALL: acute lymphoblastic leukemia

2.3. Inferring Tumor Progression from Mutational Profiles

Due to the genetic heterogeneity of tumor cells, patterns of clonal diversity within a tumor (including metastatic lesions) allow one to make inferences about the evolutionary trajectories that underlie tumor progression. Such inferences are particularly important for our understanding of the clonal origins of distant metastases because metastatic disease is the major cause of cancer-related deaths. A common way to decipher clonal relationships between primary and metastatic tumors relies on comparisons between mutational spectra and clonal composition inferred from DNA sequencing. For example, by comparing the mutations and clonal composition of a primary lobular breast cancer with those from its metastasis, which developed 12 years later, Shah et al. (62) revealed 32 new mutations in the metastatic lesion. Of these 32 mutations, only 6 were present in the primary tumor at low frequencies, whereas the others were either not detected or undetermined. This finding suggested that some of the mutations found in the metastatic lesion originated from a few clones present in the primary tumor. Ding et al. (63) provided similar results by performing whole-genome sequencing of a basal-like breast cancer and a corresponding brain metastasis that developed eight months after

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FACS: fluorescence-activated cell sorting

the initial diagnosis. A comparison between the two lesions revealed two de novo point mutations and a large deletion in the metastasis, in addition to an altered frequency of some of the common mutations. Again, these findings indicated that genomic divergence took place between the metastatic and primary tumors.

Mutational profiles can also be used to define the evolutionary origins of relapsed disease. This approach is better suited to liquid tumors, in which, in contrast to solid tumors, spatial heterogeneity does not pose an inherent barrier to the representative sampling of clonal composition. In AML, whole-genome sequencing at high coverage identified two major evolutionary pathways during relapse. In one pathway, the relapsed clones originated from the founding clone but gained additional mutations; in the second pathway, the relapsed clones arose from a subclone that survived chemotherapy and continued to evolve (10). Nevertheless, the different mutational profiles indicated that secondary tumors can arise from minor subpopulations of cells that are present in the primary tumor. Mullighan et al. (9) obtained similar conclusions in an ALL tumor by comparing gene copy number abnormalities in matched diagnosis and relapsed samples. These authors found that the clones present in relapsed disease could arise either from minor clones that were present at the time of diagnosis or from more ancestral clones that were not detected at diagnosis.

3. EXPERIMENTAL APPROACHES USED TO INFER TUMOR HETEROGENEITY

Several genome-wide methodologies have been used to investigate molecular heterogeneity between tumors. For example, transcriptomic and genomic copy number profiling has been used to investigate intertumor heterogeneity in breast and other cancer types (67–70). Molecular characterization of large breast cancer cohorts identified distinct molecular subtypes that were associated with specific clinical outcomes (67, 68). Gene-expression profiling has also been used to identify similarities between normal and cancer cell subpopulations, which predicted the probable cell of origin of some tumors (71). These genome-wide profiling methodologies, each of which has its own advantages, caveats, and limitations, have also been used to investigate intratumor heterogeneity. Profiling of bulk tumor samples or specific tumor cell subpopulations can provide useful information about the tumor as a whole but cannot determine the cellular origin of the signal, topology within tumors, or the degree of heterogeneity. However, in situ techniques preserve tissue context specificity and have been used to investigate tumor traits at the single-cell level, albeit with a low dynamic range for quantification. Single cells and specific subpopulations can also be investigated by fluorescence-activated cell sorting (FACS), and even genome-wide profiling of single cells can be performed, although there are some technical limitations (8).

Below, we enumerate some of the most common experimental approaches used to investigate tumor heterogeneity. We do not describe detailed procedures for each technique but rather provide a glimpse into their applicability and utility.

3.1. Genome-Wide Studies of Bulk Tumors

Numerous technologies using different platforms have been developed to analyze mutations, DNA methylation, gene expression, chromatin, and protein modification at the genome-wide scale and in an unbiased manner. Most of these technologies require relatively large amounts of tissue as input material, which restricts their use to analyses of bulk tissues or relatively large isolated cell subpopulations; however, these technologies have the advantage of providing quantitative data on the tumor as a whole (72). As the size of biopsy and other tissue samples available from human tumors decreases, great emphasis is being placed on the optimization of techniques for small samples (73).

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Genome-wide studies have been used to identify different molecular profiles segregated in topologically different areas of a tumor. Navin et al. (59) analyzed genomic and topologic heterogeneity in breast cancer by segmenting the tumors into different pieces, isolating the nuclei by FACS and sector-ploidy profiling, and performing array comparative genomic hybridization (aCGH) by using populations of cells with different ploidy. This experimental approach was used to define tumors as either monogenomic or polygenomic on the basis of detection of either homogeneous or heterogeneous complex clonal architecture, respectively. The presence of clonal architectures with different complexities among breast tumors was further supported by data obtained from single-nucleus sequencing of breast cancer cells (8). Notably, on the basis of analyses of different sectors of the same tumor, different clones were found to be either topologically segregated throughout the tumor or intermingled within the same sector.

Genome-wide analyses have also been used to study clonal relationships among primary and metastatic lesions. For example, in pancreatic cancer, different clones were identified in anatomically different regions of the tumor on the basis of whole-exome sequencing and copy number analysis. Furthermore, a comparison between mutations in cancer cells obtained from different areas of the primary tumor and mutations from metastatic lesions was used to identify the anatomic region within the primary tumor that probably gave rise to the metastatic clones (74). Similar approaches have been used to infer clonal selection following cancer therapy, as discussed in the following sections.

Differences in genetic aberrations have also been used to identify clonal relationships between epigenetically and phenotypically different tumor cell subpopulations. For example, by using a combination of SAGE (serial analysis of gene expression), SNP (single-nucleotide polymorphism) arrays, and FISH (fluorescence in situ hybridization) for the analysis of breast cancer cells with distinct phenotypes, Shipitsin et al. (75) observed that CD24$^+$ luminal and CD44$^+$ stemlike cell populations were genetically related but that the CD24$^+$ cells harbored additional genomic alterations, suggesting that these cells had undergone clonal divergence from CD44$^+$ cells.

FISH: fluorescence in situ hybridization

3.2. Genome-Wide Studies in Single Tumor Cells

Single cells obtained from fresh tumor samples or circulating tumor cells from peripheral blood can be used to identify genomic aberrations by whole-genome sequencing (i.e., searching for variation in numbers of aligned reads across the genome) (8), high-resolution aCGH (76), spectral karyotyping (77), and single-cell polymerase chain reaction (PCR) (78). Although single cells can also be used for whole-genome or whole-exome sequencing (79, 80), this approach still has several caveats and technical limitations (8), most notably the need for whole-genome amplification, which may introduce biases in genomic representation as well as mutations. Some of the problems associated with single-cell measurements can be avoided by using small numbers of cells instead; this approach provides sufficient input material and allows for the detection of pathways that are heterogeneously activated in single cells (81).

Isolated single cells can also be used to define variations in signaling pathways. Single-cell proteomic analyses using multiparametric measurements are useful for the identification of variations in signal transduction among single cells (49, 82). Identification of differentially active signaling pathways in single cells can provide valuable information about cellular responses to drugs, given that mutational profiles and copy number changes do not always reveal dependencies for specific cell growth and survival pathways.

3.3. In Situ Analysis of Intratumor Heterogeneity

In situ techniques are the preferred methods for investigation of intratumor heterogeneity

FFPE: formalin-fixed paraffin-embedded

IHC: immunohistochemistry

IF: immunofluorescence

for several reasons. First, because fixation and paraffin embedding of tumor samples are a routine part of diagnostic pathology, large cohorts of FFPE (formalin-fixed paraffin-embedded) samples are available for studies. Second, in situ analysis of tissue sections provides information about traits at the single-cell level while preserving tissue context. As an alternative approach, one can also isolate selected cells by laser capture microdissection to determine genetic and epigenetic alterations in discrete areas of the tumor. In situ techniques can also be applied to single cells obtained by enzymatic dissociation of tumors or to circulating tumor cells obtained from peripheral blood following fixation on a slide, although this approach does not provide information about cellular context (i.e., topology).

The detection and evaluation of antigenic markers in FFPE tissue can easily be achieved by immunodetection techniques such as immunohistochemistry (IHC) and immunofluorescence (IF). IHC is particularly useful for rapid analyses of small biopsies or whole tumor sections and for semiquantitative assessments of the expression levels of the protein of interest. For example, immunohistochemical assessment of hormone receptors and HER2 is routinely used in breast cancer diagnosis and is used to guide treatment decisions. Immunostaining of tumors allows one to easily describe immunophenotypic heterogeneity at the time of diagnosis on the basis of the intensity of expression, percentage, and location of the cells that are positive for a marker. Although immunohistochemical protocols for the simultaneous detection of two or more proteins on a single slide have been developed (83, 84), the evaluation of multiple markers on the same slide and within the same cells is more easily performed by IF. Multicolor IF is the preferred technique to investigate coexpression of proteins in the same cells, and it is useful for the identification of specific signaling pathways in defined cell subpopulations. Also, improvements in analytical systems used to identify colocalization of multiple markers in the same cell and record their coordinates have allowed investigators to map the topological locations of cell subpopulations within a tumor (85).

In situ hybridization techniques have been used to detect RNA and DNA molecules in both frozen and FFPE tissues (86–88). In situ hybridization combines molecular biology with histology and cytology or the analysis of gene expression and involves the hybridization of labeled nucleic acid probes with nucleic acids in cells. Both DNA and RNA can be used as probes, and both can be labeled with radioactive isotopes, enzymes, or fluorochromes (87, 89, 90). Immuno-FISH identifies genomic imbalances and specific DNA translocations in particular cell subpopulations by combining the hybridization of probes with the immunodetection of antigenic markers (91). Conventional FISH is also used to identify changes in messenger RNA (mRNA) expression; however, technical limitations make analyses of mRNA copy number variations less accurate. New types of mRNA probes have significantly improved analyses and quantification of spatial gene-expression patterns at the single-cell resolution level (86); such information has been used to identify cells expressing particular mRNAs in specific anatomic areas (88).

In situ PCR and in situ reverse-transcription PCR are also very valuable for detection of a particular nucleic acid in a fixed cell (92), including detection of mutations. Over the past few years, a flood of data has become available on somatic mutations in cancer genomes due to projects such as the International Cancer Genome Consortium and the Cancer Genome Atlas (93). These initiatives aim to generate large public databases that catalog genetic alterations in human cancers, which hopefully will lead to the discovery of new oncogene and nononcogene addiction mechanisms and mutations that could engender the development of new target therapies. However, the sequencing approaches and bulk tissue samples used to generate these data cannot provide information about either the number and identity of specific mutations in a given cell or the relative location of this cell within the tumors. The ability to detect somatic mutations in situ in single cells

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can, in principle, resolve these issues. Although not commonly used in clinical and laboratory practice, in situ PCR is feasible and can provide cell type–specific information about particular mutations in tumors (94–96).

4. CLINICAL IMPACT OF INTRATUMOR HETEROGENEITY

A primary consequence of intratumor heterogeneity is its impact on the accuracy of biomarker-based approaches to clinical cancer diagnosis. Depending on the context and their intended application, biomarkers can be stratified into different categories. A general classification distinguishes between biomarkers used for disease characterization (measured before therapy) and biomarkers used to measure response to therapy. Biomarkers employed for disease characterization are referred to as prognostic, predictive, and risk biomarkers, whereas biomarkers used to measure response to treatment are indicative of pharmacodynamics, efficacy, and surrogate end points (97). Biomarkers that reveal disease mechanisms are often targets for therapy and are used both to select patients who are likely to benefit from their inhibition and to monitor response to treatment (98).

Important characteristics of a good predictive biomarker are spatial homogeneity and robust expression during disease progression. However, variations in the frequency and topological location of some biomarkers within a tumor can hinder accurate cancer diagnosis and the selection of the most appropriate treatment, given that the analysis of small biopsies may not represent the tumor as a whole (**Figure 2**). Variability in biomarker expression may reflect either genetic or nongenetic heterogeneity. For example, intratumor heterogeneity for the

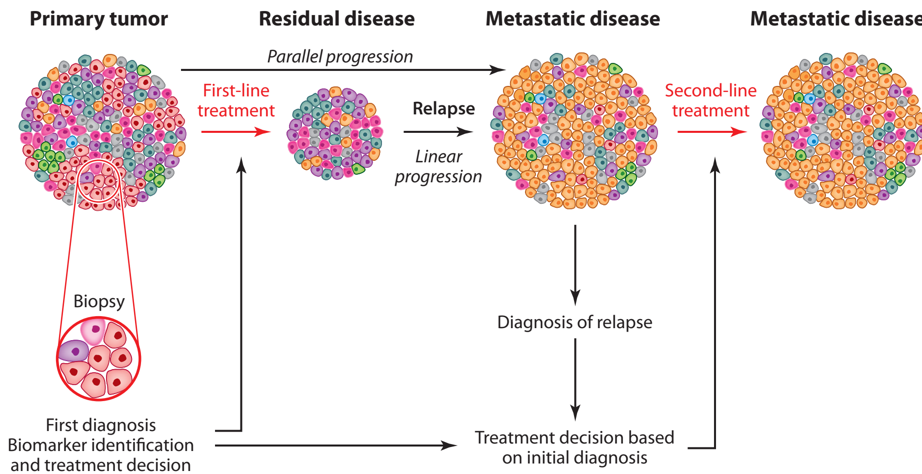

Figure 2

Effects of intratumor heterogeneity on the predictive value of biomarkers. Cancer diagnosis is usually based on sampling of the tumor by core biopsy or fine-needle aspiration that inevitably captures only a small fraction of all tumor cells and thus may not be representative of all subclones (represented by cells of different colors). The analysis of such biopsies, which probably sample the predominant clones, is used to guide treatment decisions. If successful, treatment eliminates the dominant clone, but clones that are resistant to therapy (represented by yellow cells) are positively selected and drive disease progression. Distant metastases may arise from cells that disseminated early during tumor development, before treatment or even at diagnosis, or may develop from clones that survived the initial therapy. Therefore, the clonal composition of metastatic lesions may differ significantly from that of the primary tumor sample, and treatments that are designed according to analyses of the initial diagnostic sample may be suboptimal for treatment of metastatic disease.

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Table 1 Examples of studies assessing intratumor heterogeneity and topologic clonal distribution in human tumors

Cancer type	Experimental approach and trait measured	Reference(s)
Breast	Genomic imbalance assessed by immuno-FISH in different cell immunotypes in in situ and invasive tumors	91
Breast	Sector-ploidy profiling of isolated nuclei from different areas of the tumor, representational oligonucleotide microarray analysis to identify copy number changes and chromosomal breakpoints	59
Breast	Single-cell sequencing in tumor sectors to define clonality on the basis of copy number alterations	8
Breast	Expression profiling of multiple core-needle biopsies obtained from the same patient	101
Breast	Karyotypic analysis of single cells isolated from different quadrants of the same tumor	102
Breast	Laser capture microdissection of cells from different regions in the same tumor, CGH and FISH analysis of copy number alterations	103
Breast	FISH and immunohistochemistry for *HER2* in distinct areas from the same tumor	104
Breast	*PIK3CA* mutations in different areas of the tumor	105, 155
Non–small cell lung	*EGFR* mutation status determined by primer extension assay in different areas of the same tumor	110
Cervical	Gene-expression profiling of different core biopsies from the same tumor	106
Pancreatic	Whole-exome sequencing and copy number analysis of samples obtained from different anatomic regions of a pancreatic cancer	74
Melanoma	Laser capture microdissection of cells from different anatomical areas and detection of $BRAF^{V600E}$ mutation	107
Ovarian	Quantitative multicolor immunofluorescence for *ER* and *HER2* in whole tumor sections obtained before and after chemotherapy	85
Thyroid	Laser capture microdissection of two isolated histological components and analysis of *RET* mutations and allelic losses	108
Colorectal	Gene-expression profiling of different biopsies from the same tumor	156
Colorectal	Methylation at CpG islands by bisulfite sequencing of different fragments from the same tumor	99
Soft sarcoma	Gene-expression profiles from different regions of the same tumor	109

Abbreviations: CGH, comparative genomic hybridization; FISH, fluorescence in situ hybridization.

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expression of genetic and phenotypic biomarkers and their segregation within different anatomic regions of the same tumor have been documented in multiple tumor types through the use of different experimental approaches (**Table 1**) (8, 59, 74, 85, 91, 99–110).

The limited predictive value of a single biomarker in core biopsies could be alleviated by using so-called signatures, which are obtained through simultaneous measurements of many markers that provide a more robust measure of a tumor's biologic potential. Several studies have addressed the influence of intratumor heterogeneity on the precision of microarray-based classifiers and have demonstrated that gene-expression profiling can be used to identify gene sets with low intratumor variability and better predictive value (101, 106, 109).

Because biomarkers reflect the biological properties of tumors, they are susceptible to changes during disease progression (30). This susceptibility poses a challenge for adjuvant targeted therapies and therapeutic strategies for relapsed disease because they are often chosen on the basis of the initial diagnosis of the primary tumor, under the assumption that the target is maintained during disease

progression. Divergent evolution of metastatic tumor cells and different microenvironments at the metastatic sites could contribute to the change of expression of the biomarkers that were initially identified in the primary tumor. Therefore, treatment of metastatic disease according to the biomarkers expressed in the primary tumor may not always be optimal. Ideally, therapeutic strategies for the treatment of metastatic disease would be designed on the basis of new diagnostic biopsies. However, it is impractical to obtain biopsies from some anatomic locations; thus, the predictive value of biomarkers assessed in the initial diagnostic specimens is critical for the clinical decision-making process (16). Therefore, for optimal benefit from adjuvant therapies, predictive biomarkers should be homogeneously expressed in the tumor (to reduce sampling bias during diagnosis) and expressed in relapsed and metastatic disease. Moreover, the frequency and prevalence of predictive biomarkers should be defined and validated in the context of inherent tumor heterogeneity.

4.1. Heterogeneity in Expression of Biomarkers within Tumors

Here, we discuss how intratumor variations in phenotypic or genetic biomarkers present problems for cancer diagnostics and the ability to predict response to treatment. According to several studies, some biomarkers used to predict response to treatment display substantial spatial segregation within tumors; consequently, there is a lack of concordance among core biopsies obtained from different areas of the same tumor (**Table 1**). For example, in breast cancer the amplification of the *ERBB2* oncogene at chromosome 17q21 is used as a selection criterion for the administration of trastuzumab, a monoclonal antibody against the HER2 protein (111). In some tumors, strong expression of HER2 (defined by IHC) is used as a surrogate marker for *ERBB2* amplification. However, topological segregation of cells with and without *ERBB2* overexpression has been found in breast tumors (112). Another example of a biomarker with heterogeneous distribution is *K-RAS* mutation, which is used to predict (*a*) resistance to the anti-EGFR (epidermal growth factor receptor) therapies cetuximab and panitumumab in colorectal cancer (113, 114) and (*b*) expression of hormone receptors (estrogen and progesterone receptors) in breast cancer (115, 116).

The problem of intratumor heterogeneity is also reflected in the discordant diagnosis that is sometimes observed between biopsies and surgically resected tumors. In non–small cell lung cancer (NSCLC), the presence of mutations in EGFR is used as a predictive biomarker for gefitinib response (117). Analyses for EGFR mutations in different biopsies obtained from the same tumor showed intratumor heterogeneity for EGFR mutational status and a high discordance between paired core biopsies and resected tumors (118). Intratumor heterogeneity for HER2 and discordance between biopsies and surgically removed tumors have also been described in gastric cancer (119).

Spatial heterogeneity in the expression of diagnostic markers is not limited to regional variability; some biomarkers display a cell-to-cell "mosaic" pattern of variation. Allison et al. (120) reported that in a series of 1,329 breast cancer cases, 23% exhibited HER2 heterogeneity, which in most of the cases consisted of neighboring cells with different centromeric probe and HER2 signals. So-called mosaiform and regional heterogeneity in the expression of receptor tyrosine kinases (RTKs) has also been observed in glioblastoma (121, 122), a disease in which amplification of RTKs has been implicated in driving disease progression. Coamplification of RTKs in the same glioblastoma is very frequent, but coamplification of two RTKs in the same cell seems to be a rarer event. Instead, mosaic amplification of RTKs in intermingled cells is common. Although all the cells are derived from a common precursor, the mutually exclusive amplification of RTKs in individual glioblastoma cells and their coexistence within the same tumor suggest the possibility of oncogenic codependency associated with a distinct selective advantage (122).

EGFR: epidermal growth factor receptor

NSCLC: non–small cell lung cancer

RTK: receptor tyrosine kinase

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PET: positron emission tomography

4.2. Discordance for Biomarkers in Distant Metastases

Progression to metastatic disease has traditionally been viewed as the end stage of a linear tumor progression initiated by a subset of cancer cells in advanced-stage tumors. However, recent data in animal models and in cancer patients challenge this view and imply that metastatic dissemination may occur early in the disease course, thereby leading to the parallel progression of primary and metastatic tumors (123). In both scenarios, primary and metastatic tumors could evolve independently and acquire different phenotypes. Also, differences in the tumor microenvironment at the primary and metastatic sites could also favor the acquisition of different phenotypic features (124).

The discordance in predictive biomarkers between primary and metastatic tumors implies that targeted therapies based on the assessment of biomarkers in the primary tumor at the time of diagnosis may be ineffective in metastatic lesions. The discordance in biomarkers at different anatomic locations has been described in NSCLC, wherein some lymph node metastases lacked the *EGFR* mutation detected in the primary tumor (reviewed in Reference 118). Several studies have revealed a discordance in *ERBB2* amplification between the primary tumor and metastases in breast cancer (125, 126); both loss and gain of *ERBB2* amplification were observed during metastatic progression.

4.3. Additional Diagnostic Challenges That Arise from Intratumor Heterogeneity

As mentioned above, inadequate tumor sampling in tumors with heterogeneous expression of biomarkers could cause interpretative errors at diagnosis and suboptimal therapeutic decision making. Such uncertainty highlights the need to establish robust standard diagnostic criteria. As an example, according to ASCO (American Society of Clinical Oncology) guidelines, genetic heterogeneity in the *ERBB2* gene should be reported "if there are more than 5% but less than 50% of infiltrating tumor cells with a ratio higher than 2.2" or "if there are more than 5% but less than 50% of infiltrating tumor cells with more than 6 HER2 signals per cell" (127, p. 611). This new guideline aims to decrease subjectivity among pathologists at the time of diagnosis. However, the clinical significance of heterogeneity for the *ERBB2* gene and its potential influence on treatment outcome is still unknown, and on the basis of the above-described criteria, tumors harboring relatively infrequent cells with HER2 amplification are not considered amplified for treatment decisions (120). Therefore, accurate and standardized descriptions of biomarker heterogeneity at the time of diagnosis could provide valuable information to guide treatment decisions. In fact, patients with tumors displaying heterogeneity for *HER2* amplification are associated with a shorter disease-free survival (128), which supports the idea that measurements of heterogeneity for biomarkers can provide clinically relevant information.

Imaging technologies such as positron emission tomography (PET) are commonly used to diagnose cancer and monitor disease progression. However, spatial heterogeneity in tumor cell metabolism can cause diagnostic errors. For example, in NSCLC patients tumor-diameter measurements based on PET underestimate tumor size, compared with measurements obtained by computed tomography scans, because of variation in glucose uptake at different areas of the tumor (129).

Another important diagnostic consideration is sample size. Typically, diagnostic specimens are core-needle biopsies or fine-needle aspirates, which limit ancillary biomarker tests to those that can be performed on small amounts of tissue. As discussed above, biopsies usually do not provide enough input material for high-throughput genomic analyses. Instead, they are suitable for in situ multiparametric analysis or certain molecular diagnostic tests based on a few markers. The identification of clinically

relevant recurrent mutations among tumors could be used to simplify analyses of genetic alterations to a limited number of mutations. For example, Sequenome's MassARRAY® OncoCarta Panel provides a molecular snapshot of the mutational profile of 238 mutations across 10 oncogenes; this technology is now used to guide cancer classification and treatment decisions in several cancer centers (130). The assessment of multiple predictors in situ could also provide more accurate measurements of outcome and response. However, this approach poses a technical challenge because the design and validation of multiparametric measurements of biomarkers will require normalization and standardization by means of reproducible protocols and implementation of automated technology.

Intratumor heterogeneity can be assessed by use of FFPE samples and many different analytes, including proteins, mRNA, DNA, and cellular and tissue morphology, either alone or in combination. To translate multiparametric measurements into clinical use, one must first know which parameters provide clinically relevant information (**Figure 3**). Multiple readouts can be assessed by retrospective investigation of large cohorts of archived tissues with high-throughput imaging and morphometric quantification platforms. The application of pattern-recognition programs for the interpretation of immunohistochemical staining has contributed to a decrease in interpretative bias and is revolutionizing the way we analyze phenotypic heterogeneity in large numbers of samples. However, such automated analyses still require better standardization of cutoffs and the definition of scoring ranges for each marker. Moreover, these measurements must be combined with mathematical approaches and bioinformatic tools to provide a numeric estimation of their degree of expression or coexpression, as well as their degree of heterogeneity (91, 131). Future studies will inform the associations between heterogeneity and clinical information, such as drug response, risk of metastasis, and outcome, and in doing so will promise to identify measurements that can be used as clinically relevant biomarkers. In addition to improving the validation of biomarkers, the introduction of robust measurement criteria will decrease intra- and interobserver variability within diagnostic laboratories.

4.4. Tumor Evolution and Therapeutic Resistance

Intrinsic factors (e.g., genetic instability, mutational rate, and epigenetic status) and extrinsic factors (e.g., microenviromental factors and therapy) that shape intratumor heterogeneity also influence therapeutic responses by creating tumors with a higher diversity of phenotypes for selection to act on. Most cancer therapies fail to completely eliminate all of the tumor cells, and the tumor cells that grow out after chemotherapy and radiation often display greater genetic instability or emergent biological properties that lead to resistance. An increase in genomic instability per se does not provide a selective advantage to cancer cells; in fact, excessive genetic instability could become deleterious by exceeding the threshold for viability. However, under certain selective pressures the presence of a mutator phenotype and the genetic plasticity induced by genomic instability could be favored. For instance, the anticancer effects of chemotherapy rely on the generation of DNA damage, which can activate checkpoint and DNA-repair mechanisms that slow down the replication rate of cancer cells. Therefore, during tumor progression and following chemotherapy, cells harboring inactivating mutations in genes involved in nucleotide excision repair, such as *BLM* (Bloom's syndrome gene) and mismatch-repair genes, could be positively selected because alterations in these genes increase the mutational rate of the tumor, thereby providing a higher cellular diversity for selection forces to act on (132–136). In this scenario, genetic instability and the acquisition of mutations in the DNA mismatch-repair system can provide a selective advantage even in the presence of drugs

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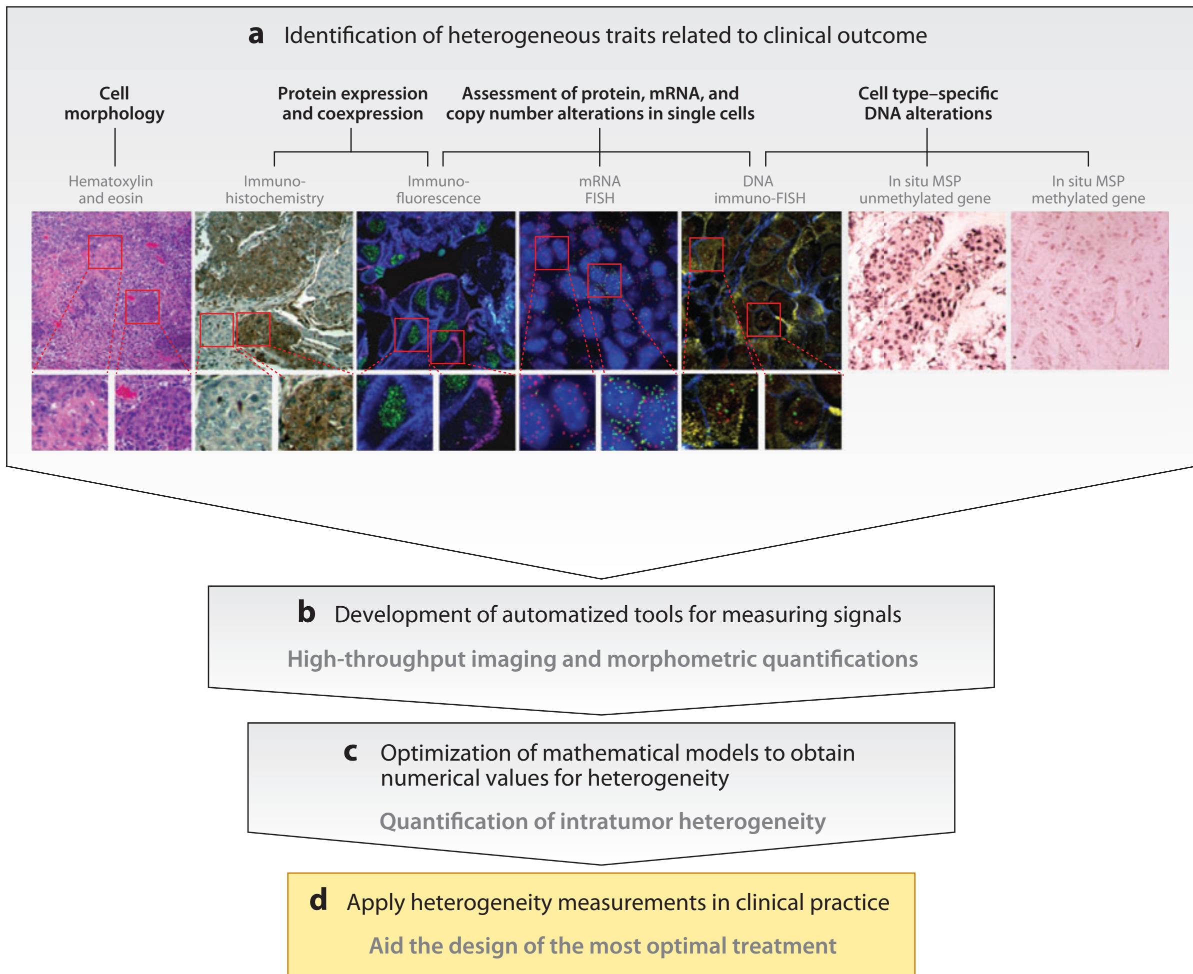

Figure 3

Proposed steps in enabling multiparametric in situ analysis of biomarkers in tumor samples. (*a*) Identification of biomarkers in formalin-fixed paraffin-embedded tissues, which are in wide clinical use and preserve cell-to-cell heterogeneity. To validate and incorporate intratumor heterogeneity measurements into diagnostic practice, one must identify tumor traits that provide clinically relevant information. Possible analytes include morphology, proteins, DNA, and RNA, alone or in combination. (*Left to right*) Standard morphology; immunohistochemical staining for PTEN; immunofluorescence staining for CD44 (*blue*), CD24 (*pink*), and phospho-STAT3 (*green*); messenger RNA (mRNA) fluorescence in situ hybridization (FISH) for *HES1* (*red dots*) and the Wnt pathway target *MYC* (*green dots*) (reproduced courtesy of Dr. Shalev Itzkovitz, Massachusetts Institute of Technology); immuno-FISH for CD44 (*blue*), CD24 (*yellow*), and 8q24 (*red*), along with a centromeric probe for chromosome 8 (*green*); in situ methylation–specific polymerase chain reaction (MSP) showing the detection of unmethylated and methylated DNA for the *SCGB3A1* (*HIN-1*) gene. (*b*) To standardize the evaluation and quantification of multiple parameters, one must develop automated tools that reliably measure multiple signals with high accuracy. (*c*) On the basis of these measurements, a numerical value of the degree of heterogeneity can be defined using bioinformatic and mathematical models, which (*d*) can ultimately be utilized during the clinical decision-making process.

(137, 138). For example, the use of temozolomide in glioblastoma multiforme favors the positive selection of cells harboring *MSH6* inactivating mutations that are causally associated with temozolomide resistance and the acquisition of a hypermutator phenotype (139).

In other cases, therapies influence tumor progression by favoring the clonal selection

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of cells with phenotypes that confer resistance to treatment. For example, anthracyclines in breast cancer therapy favor the selection of cells with overexpression of *LAPTM4B* and *YWHAZ*, which contribute to de novo chemoresistance by sequestering the drug and by activating antiapoptotic mechanisms (140). In lung cancer, amplification of *MET* confers resistance to the EGFR inhibitor gefitinib by activating the ERBB3 pathway (141).

Targeted therapies frequently favor the selection of cells that harbor additional mutations in the target proteins. For instance, in *BRCA1/2* mutant breast and ovarian cancers with impaired homologous recombination, poly(ADP-ribose) polymerase (PARP) inhibition is a potential therapeutic strategy (142). Development of resistance to PARP inhibitors can result from the acquisition of intragenic deletions in *BRCA2* that restore its DNA-repair function (143). The EGFR inhibitor gefitinib is used in NSCLC to target mutated *EGFR*. Mechanisms of resistance include the positive selection of cancer cells with secondary *T790M* mutations in *EGFR* that confer insensitivity to gefitinib (144). Resistance to the BCR-ABL inhibitor imatinib mesylate in chronic myeloid leukemia (CML) can emerge as a consequence of acquiring secondary mutations in the ABL kinase domain, such as a threonine-to-isoleucine amino acid substitution (T315I) that confers insensitivity to imatinib (145). Therapeutic resistance can also arise from mutational activation of alternative signaling pathways that provide prosurvival signals following inhibition of pathways targeted by therapies, such as mutations in *K-RAS* that confer resistance to the B-RAF inhibitor PLX4032 in melanomas with mutant *B-RAF*^{V600E} (146).

Unraveling the pathways of tumor evolution and resistance could shift therapeutic strategies toward up-front prevention of outgrowth of resistant clones. For example, cells with secondary mutations in BCR-ABL in CML are present as minor subpopulations in the initial disease (147, 148), and cells with *MET* amplification are also found in NSCLC with mutant *EGFR* prior to therapy (149). In principle, detection of such clones at diagnosis could cause changes in treatment regimens that are designed to suppress their growth. Such changes would require identification of rare subclones before the start of the treatment, which would be challenging, given the small samples used for diagnosis and technical limitations, as well as the development of new drugs that target these mutated proteins. Nevertheless, detection of rare subclones within tumors will undoubtedly become increasingly possible during the next few years thanks to the improvements in sequencing technologies and bioinformatic analysis tools and further drops in the price of sequencing. Furthermore, functional genomic approaches will provide excellent opportunities for the discovery of pathways involved in drug resistance, which should provide both new biomarkers and additional rational therapeutic targets.

PARP: poly(ADP-ribose) polymerase

5. MEASUREMENTS OF INTRATUMOR HETEROGENEITY AS A BIOMARKER

High genetic and phenotypic diversity in tumors provides a more expansive substrate for adaptive responses to selective pressures such as hypoxia, metabolic stress, chemotherapy, and radiotherapy (133). Therefore, the genomic and phenotypic variation among tumor cells and the degree of intratumor heterogeneity may themselves be a prognostic factor. Measurements of genetic diversity can provide information about the underlying dynamics of cancer progression. Maley et al. (12) investigated genetic diversity in Barrett esophagus, a premalignant lesion, by analyzing differences in DNA content, loss of heterozygosity, microsatellite shifts (new alleles), and *CDKN2A* or *TP53* mutations among cells. They measured diversity by adapting mathematical models used in ecology to estimate the diversity in ecosystems, such as the Simpson and Shannon indexes, and they found that the measurement of diversity based on any trait could predict progression to invasive cancer. Using similar experimental approaches, Park

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et al. (91) demonstrated that the degree of diversity in particular cell subpopulations correlates with clinicopathological features of breast tumors. Teixeira et al. (102) also found that the numbers of genomic imbalances in primary breast cancers correlated with the presence of lymph node metastases. Therefore, measurements of genetic and phenotypic variation could provide valuable diagnostic and risk-stratification tools if included in clinical practice as predictive biomarkers (128).

The degree of intratumor heterogeneity can also be inferred from certain tumor properties. A potential source of genetic heterogeneity is CIN; therefore, measurements of CIN could be used as surrogate biomarkers for genetic heterogeneity. For example, a CIN score derived from aneuploidy-related genes has predictive value in multiple cancer types (150). However, differences in DNA content among cells, caused by unequal distribution of chromosomes during mitosis, indicate CIN. Measures of DNA content by flow cytometry have been used to estimate the stem-line scatter index, which classifies malignant aneuploid, diploid, and tetraploid tumors into low- and high-grade subtypes (151).

6. MODIFYING INTRATUMOR HETEROGENEITY TO IMPROVE TREATMENT OUTCOMES

Intratumor heterogeneity is not a static characteristic but rather a dynamic property arising from cellular adaptation and constant clonal selection. A primary consequence of intratumor heterogeneity during treatment is the inevitable selection of preexistent unresponsive clones. In the case of targeted therapies, the mechanism of resistance often involves secondary mutations in the target, the activation of compensatory survival pathways, or the positive selection of clones that do not express the target. Downregulation of the target by epigenetic mechanisms is also a mechanism of resistance to therapy (152, 153). For instance, the NY-ESO-1 antigen is used in ovarian cancer for immunotherapy, but response to this treatment is limited due to heterogeneous expression among and within tumors. The intratumor expression of NY-ESO-1 varies depending on promoter methylation, and induction of DNA hypomethylation with azacitidine treatment can restore its expression in nonresponder cells (152). Similarly, heterogeneity in the expression of cancer/testis antigens in human melanoma can be restored by azacitidine treatment that increases the efficacy of immunotherapy (153). These results suggest that DNA hypomethylating and other epigenetic modifying compounds could be useful to restore the expression of targets that are silenced during disease progression.

Epigenetic mechanisms of chromatin remodeling mediate cellular adaptation to chemotherapy (53). This finding suggests that the blockade of epigenetic adaptation can also be used therapeutically to impede the development of chemoresistance.

7. CONCLUDING REMARKS

Intratumor heterogeneity and its evolution during tumor progression pose a major challenge for effective cancer therapy. Regardless of its underlying mechanisms, heterogeneous expression of existent biomarkers has important implications for both diagnostic accuracy and treatment success. Going forward, it will also have a profound effect on the discovery and validation of new predictive biomarkers, which are necessary for effective implementation of personalized cancer therapy and the identification of new therapeutic drug targets. More cost-effective strategies for evaluating the efficacy of new therapeutic compounds and improved patient stratification, according to the likelihood of response to treatment, also require robust validated biomarkers (**Figure 4**), the identification and validation of which are confounded to some degree by the phenomenon of tumor heterogeneity. Thus, incorporating measurements of intratumor heterogeneity during the validation of biomarkers will be necessary to increase their predictive value and accuracy.

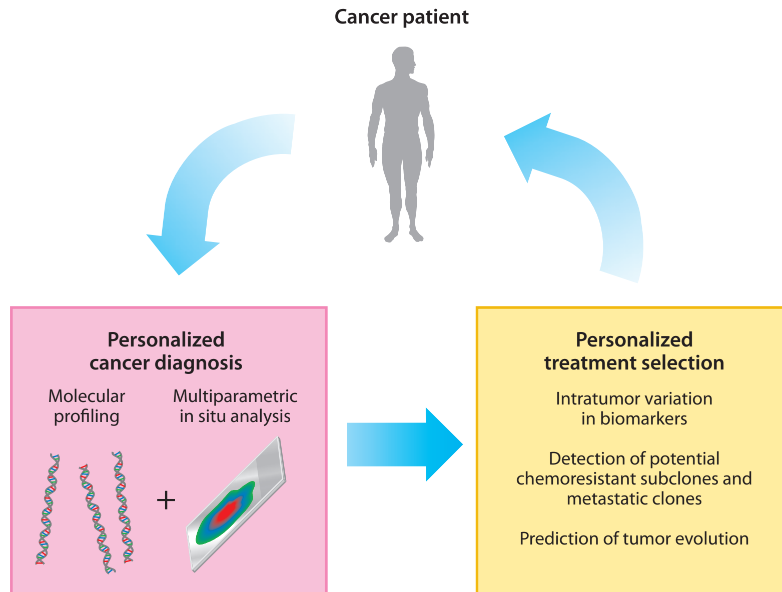

Figure 4

Proposed strategy for incorporation of intratumor heterogeneity measurements into clinical practice. Following the identification of malignant disease, personalized diagnosis will be accomplished by investigating different aspects of the tumor through the use of molecular approaches and in situ analyses. Robust biomarkers that accurately reflect intratumor heterogeneity will provide better predictive estimates of therapeutic responses and putative pathways of tumor progression. All these data will be integrated to define the best treatment for each individual tumor.

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SUMMARY POINTS

1. Heterogeneity in tumor cell phenotypes is a multifaceted phenomenon that arises from the interplay between genetic heterogeneity and the nongenetic factors that shape cellular phenotypes. Darwinian evolution, which underlies the progression of cancer, creates a unique complex clonal architecture in every individual tumor.
2. Nongenetic heterogeneity in cancers cannot be reduced to differentiation hierarchies; additional factors, including stochastic cellular heterogeneity, need to be considered.
3. Resistance of tumors to therapy can arise from both heritable and nonheritable phenotypes, but the relapse of an initially sensitive tumor is driven by the outgrowth of genetically distinct cells or by cells with stable, epigenetically distinct traits.
4. Intratumor phenotypic and genetic heterogeneity could cause spatial variability in the expression of clinically important biomarkers. Because cancer diagnosis relies on biopsies that sample only a small fraction of a tumor, such heterogeneity can substantially hamper the predictive power of biomarkers.
5. High levels of genetic heterogeneity are strongly associated with poor clinical outcomes, which could make genetic diversity a useful clinical marker. The most likely underlying mechanism is that higher diversity increases the "evolvability" of a tumor, given that genetic diversity is a substrate for Darwinian evolution.

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Annual Review of Pathology: Mechanisms of Disease

Volume 8, 2013

Contents

Annu. Rev. Pathol. Mech. Dis. 2013.8:277-302. Downloaded from www.annualreviews.org
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Pathogenesis of Langerhans Cell Histiocytosis
Gayane Badalian-Very, Jo-Anne Vergilio, Mark Fleming, and Barrett J. Rollins 1

Molecular Pathophysiology of Myelodysplastic Syndromes
R. Coleman Lindsley and Benjamin L. Ebert 21

The Role of Telomere Biology in Cancer
Lifeng Xu, Shang Li, and Bradley A. Stohr 49

Chromosome Translocation, B Cell Lymphoma, and Activation-Induced Cytidine Deaminase
Davide F. Robbiani and Michel C. Nussenzweig 79

Autophagy as a Stress-Response and Quality-Control Mechanism: Implications for Cell Injury and Human Disease
Lyndsay Murrow and Jayanta Debnath 105

Pathogenesis of Antineutrophil Cytoplasmic Autoantibody–Associated Small-Vessel Vasculitis
J. Charles Jennette, Ronald J. Falk, Peiqi Hu, and Hong Xiao 139

Molecular Basis of Asbestos-Induced Lung Disease
Gang Liu, Paul Cheresh, and David W. Kamp 161

Progressive Multifocal Leukoencephalopathy: Why Gray and White Matter
Sarah Gheuens, Christian Wüthrich, and Igor J. Koralnik 189

IgA Nephropathy: Molecular Mechanisms of the Disease
Jiri Mestecky, Milan Raska, Bruce A. Julian, Ali G. Gharavi, Matthew B. Renfrow, Zina Moldoveanu, Lea Novak, Karel Matousovic, and Jan Novak 217

Host Responses in Tissue Repair and Fibrosis
Jeremy S. Duffield, Mark Lupher, Victor J. Thannickal, and Thomas A. Wynn 241

Cellular Heterogeneity and Molecular Evolution in Cancer
Vanessa Almendro, Andriy Marusyk, and Kornelia Polyak 277

The Immunobiology and Pathophysiology of Primary Biliary Cirrhosis
Gideon M. Hirschfield and M. Eric Gershwin 303

Digital Imaging in Pathology: Whole-Slide Imaging and Beyond
Farzad Ghaznavi, Andrew Evans, Anant Madabhushi, and Michael Feldman 331

Pathological and Molecular Advances in Pediatric Low-Grade Astrocytoma
Fausto J. Rodriguez, Kah Suan Lim, Daniel Bowers, and Charles G. Eberhart 361

Diagnostic Applications of High-Throughput DNA Sequencing
Scott D. Boyd 381

Pathogenesis of the Viral Hemorrhagic Fevers
Slobodan Paessler and David H. Walker 411

Skeletal Muscle Degenerative Diseases and Strategies for Therapeutic Muscle Repair
Mohammadsharif Tabebordbar, Eric T. Wang, and Amy J. Wagers 441

The Th17 Pathway and Inflammatory Diseases of the Intestines, Lungs, and Skin
Casey T. Weaver, Charles O. Elson, Lynette A. Fouser, and Jay K. Kolls 477

Indexes

Cumulative Index of Contributing Authors, Volumes 1–8 513

Cumulative Index of Article Titles, Volumes 1–8 517

Errata

An online log of corrections to *Annual Review of Pathology: Mechanisms of Disease* articles may be found at http://pathol.annualreviews.org

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