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Review Article

The Importance of Heterogeneity in Tumor Pathology

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Summary: Heterogeneity of cell, tissue, and tumor genotype, form, and function confounds clinicopathologic research and therapeutic strategies. Its significance and importance usually are underestimated and underreported, and there is no consistent statistical or biomathematical framework for its interpretation. New technologies for the quantitative study of pathology specimens and a growing awareness of the clinical importance of biodiversity within individual tumors and populations of tumors herald new approaches to the problems posed by dynamic structural and functional complexity in human tumors. **Key Words:** Heterogeneity–Human tumors–Laser cytometry–Clinical pathology.

Editor's note: *David Rew is no novice in the molecular aspects of anatomic pathology. In this clear exposition of heterogeneity in neoplasia and its impact on diagnostic, prognostic, and therapeutic research, he places us better to deal with contemporary research data. More than that, he prepares us for the next century's advances. The title of his earlier publication on tumoral heterogeneity*—Heterogeneity, Biodiversity and Bioperversity in Human Solid Tumors—*is not only catchy, but accurate.*

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The 20th century has been the century of cell and molecular biology and of systematic clinical pathology. However, despite all of the remarkable advances, solid tumors often defy treatment, and the biology of aggressive malignancy is not well understood. Pathologists have a key role to play in the study of histologic material to discover better prognostic markers and (much more importantly) the mechanisms for therapy.

The measurement of the expression of one or another biologic markers is a common theme in modern clinicopathologic research. Data may be correlated with indices of clinical behavior such as time to recurrence, response to therapy, or survival. The complexity inherent in even the simplest neoplastic structure often is hugely underestimated in the search for clear descriptors, classifications, and quantitative biomarker measurements. The issue of heterogeneity rarely features highly in the research literature, although there have been recent efforts to redress the balance, notably within the cytometry community (1). We need to recognize the extent of the problem posed by cell and tissue heterogeneity and to confront this complexity with new intellectual stratagems and technologies.

Heterogeneity may be defined as diversity or as composition by diverse elements not having a uniform quality throughout. This complexity is a fundamental feature of biologic systems and is a major impediment to the understanding of the behavior of solid neoplasms at the tissue, cellular, genetic, tumor, and organ levels. There are two key clinical consequences of heterogeneity. The first is that it complicates the interpretation of clinicopathologic and prognostic research. The second is the effect that it has on the response of tumors to adjuvant treatment. The forms of heterogeneity are diverse. This article discusses the categories of heterogeneity of form and function that confound cancer research and therapy (Table 1).

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TABLE 1. *Forms of heterogeneity in tumor biology*

Form of Heterogeneity	Implications
Structural heterogeneity	Confounds interpretation of biomarker expression; creates abnormal physiology, e.g., hypoxia, acidity
Genotypic heterogeneity	Allows natural selection of abnormal clones; predisposes to the emergence of neoplasia; accounts for variations in biology of metastases; allows emergence of resistance to therapy
Chromosomal disorder	Accounts for aneuploidy; creates abnormal genotypes; may predispose to neoplasia
Behavioral heterogeneity	Manifests as variation in critical cell functions between and within tissues and tumors, e.g., proliferation, apoptosis; obscures biologic potential of tumor cell clones
Population heterogeneity (geographic, racial, social, epidemiological)	Manifests as different individual susceptibility to cancer; underlines importance of clinical trials, large population samples, cohort studies
Temporal heterogeneity: seconds, minutes, hours (short term)	Key cell and molecular events obscured by rapid turnover (e.g., regulatory protein function, apoptosis, mitosis)
Temporal heterogeneity: days, weeks (intermediate term)	Changes in tumor biology, such as growth fraction, vascularity, resistance to treatment
Temporal heterogeneity: months, years (long term)	Cumulative genomic damage in tissues. May include the timescale for development of individual tumors
Apparent heterogeneity	Attributable to cell cycle and cell population dynamics
Artifactual heterogeneity	Attributable to experimental technique, preparation of samples, sensitivity of measurement by instruments
Heterogeneity of defences against adjuvant treatment	Determine variable response to treatment; aid evolution of cytotoxic drug and radioresistance
Complexity of technology	Confounds choice of tools to study tumor biology; affects quantitation and sensitivity of measurements

STRUCTURAL HETEROGENEITY

The most obvious form of heterogeneity in pathology is that which can be observed even at low resolution through the microscope. The observer can readily appreciate spatial and structural heterogeneity in all tumors in the stained tissue section. Most epithelial tumors display considerable variation in cellular morphology and tissue architecture from one microscope field to the next, for example in the proportions of tumor cells, stromal cells, and supportive elements of various lineages.

The macroscopic, three-dimensional architecture of tumors also is heterogeneous. In many tumors, this manifests as a proliferative face or edge, with hypoxic or necrotic areas elsewhere. An example of this can be clearly seen in a large liver metastasis or colorectal tumor. Thus, the site sampled may be of considerable importance if representative data on proliferative activity is to be obtained. From what sites within the tumor (the growth face, center of tumor) should measurements be obtained and in what proportions?

GENETIC HETEROGENEITY

Patients with cancer have genetic differences between the parent genotype and the tumor. These differences vary with time, with growth, and with site within the tumor. It is now clear that the genetic abnormality is not a single switch or event that converts the normal to the malignant state but is a randomly staged and triggered progression of genetic and functional changes in the cell. These may be accelerated by environmental factors, such as background radiation, and by hereditary genetic changes, such as in colorectal cancer progression and the familial adenomatous polyposis phenotype (2).

Single genes display considerable mutational heterogeneity within populations. For example, more than 2000 point mutant variants of the p53 gene have been detected (3). Many of the mutations may be functionally neutral. Other cumulative point mutations in DNA may lead to increasing genetic instability and to the production of dysfunctional proteins. Genetic disorder also may arise in chromosomes through a number of different mechanisms, including gene loss, gene amplification, and DNA methylation. One morphologic manifestation of this genetic heterogeneity is the state of aneuploidy, which is found in a large proportion of solid tumors (4). These disorders give rise to pluripotentiality of biologic function and biomarker expression. In addition, there is no one point at which a tumor can be considered to have achieved its definitive genotype. Genetic diversification, such as that manifested by heterogeneity of ploidy, continues throughout the life of a tumor (5).

TEMPORAL HETEROGENEITY

All biologic systems change with time. Biologic processes change during short and long periods of time. In

the former case, many biomarkers are key enzymes or functional proteins (growth factor receptors, oncoproteins) that act dynamically and that can turn over rapidly (seconds, minutes, or hours) within the cell. Thus, inspection of a tumor fixed at one time point may not be at all representative of its immediate history or future potential. We cannot be certain that heterogeneity in space and time for any one parameter within a tumor is any less or greater than intertumor variation for the same parameter. Time-dependent events usually cannot be deduced from static techniques of biologic measurement.

During longer time periods (days to weeks), the physical and functional characteristics of a tumor change significantly. Tumor growth is the product of an imbalance between cell production and cell loss, which varies considerably with time and changing environmental factors. Tannock (6) has described this infinitely varying status of the composition and behavior of a tumor as the "heterogeneity of heterogeneity," thus recognizing that the heterogeneous characteristics of any tumor may change continuously in place and time.

The passage of time also confers heterogeneity upon cell populations through the process of aging. Cell replication confers age on the cell during mitosis through the loss of repetitive DNA from the telomeres at the ends of chromosomes each time a cell divides. This mechanism is believed to impose a limit on the number of divisions that a cell can make before the replicative mechanism is terminally damaged. In the presence of the enzyme telomerase, stem and malignant cells may be protected from DNA loss and become effectively "immortalized," as in testicular germ line cells. A tumor population may possess considerable heterogeneity of replicative potential, in part attributable to differences between cells in telomerase expression and function (7).

HETEROGENEITY OF CRITICAL CELL FUNCTIONS

Heterogeneity is found in the biology of critical cell functions. Such processes include proliferation, apoptosis, intra- and intercellular signalling, cell adhesion, angiogenesis, and metastatic behavior, usually under the control of regulatory genes. Many specified genes and their protein products have been implicated in the sequence of events leading from normal cell behavior to malignancy, as oncogenes, tumor promoters, and suppressors. Examples include *fos*, *ras*, c-*erb*B2, c-*myc* and p53 (8–10).

The cell cycle model can be used to interpret many of the apparent complexities of cell and tissue heterogeneity. All cell populations are a complex mixture of proliferating, quiescent, and terminally differentiated cells. The proportion of cells capable of proliferation in the population is the growth fraction. This may approach 100% in a rapidly proliferating tissue. These populations are asynchronous, such that varying numbers of cells will be quiescent or in any phase of the cell cycle at any one time. A homogeneous population, such as a tumor cell line in culture, may appear heterogeneous for the expression of a cell cycle-dependent marker at any one time point. Thus, cell cycle asynchrony in complex cell populations such as tumors imposes apparent heterogeneity of biomarker expression on a population of otherwise identical cells.

Levels of many measurable gene products vary between quiescent and proliferating cells and fluctuate through the cell cycle with DNA synthesis and cell division. These events are under the control of regulatory mechanisms, check points, and switches, including the cyclins and cyclin dependent kinase families of proteins. Thus, key functional enzymes, regulatory proteins, and membrane receptors are likely to be expressed in all cells but at varying levels through the cell cycle.

The measurement of proliferation markers provides examples of how heterogeneity complicates data interpretation in a number of ways. Many markers have been assessed, including the mitotic index; the flow cytometric S-phase fraction; thymidine analogue labeling in vitro and in vivo; and cell cycle-associated proteins, such as Ki67 and proliferating cell nuclear antigen.

Positional Heterogeneity

It will be important to know whether a tissue section under study derives from the proliferative face or the necrotic center. Intratumor variation for such markers may exceed the intertumor variation, thus devaluing population-based prognostic studies of tumor markers.

Cell Cycle Asynchrony

Less than 10% of cells may be in the S-phase at any one time in an asynchronous but otherwise homogeneously proliferating tumor cell population. Single, "static" measurements of a proliferation marker will yield a labeling index that does not identify the entire population of proliferative cells. The integrated measurement of temporal accretion of a chosen marker will yield different results to a snapshot analysis. Thus, in the case of cell proliferation, continuous infusion of a tumor with a halogenated pyrimidine thymidine analogue such as bromodeoxyuridine labels all cells as they come into cycle and identifies a much larger proliferative population, the growth fraction, than does flash or pulse labeling (11, 12).

Temporal Heterogeneity

Proliferative potential and growth fraction change with time, size, vascularity, and certainly with location in a tumor such that the proliferative biology in one part of an early, unconstrained, well-vascularized early invasive tumor may bear no comparison with any part of large, hypoxic, aging tumor of similar lineage.

HETEROGENEITY, BIOLOGIC AGGRESSIVENESS, AND METASTASIS

The characteristics of tumor cells may be heterogeneous for biologic aggressiveness. It is not possible to be certain which cells or microscopic fields in a tumor possess the greatest proliferative, apoptotic, invasive, or metastatic potential. Features analyzed in a clinicopathologic series may not be representative of biologic capability. The actions of individual, rogue cells may be more important than are the median behavioral traits of the whole population in shaping events.

Metastasis is the primary process that determines the aggressiveness of the tumor and the clinical outcome. There may be considerable heterogeneity of genetic content, form, function, and composition between primary tumors and their metastases, which cannot necessarily be regarded as simple clones of their parent primary tumors (13). Metastases develop in a different immunologic or nutritional environment, such as in a lymph node, liver, or bone marrow, which influences their form and structural heterogeneity. For example, a liver metastasis is not spatially confined to the same growth surface as its parent primary intestinal tumor.

Much clinicopathologic research is undertaken on samples from the primary tumor. However, once the tumor has been resected, its constituent cells can no longer influence outcome. The clinical progression is determined by the metastases. Thus, correlative studies of biomarkers in surgically resected primary tumors and clinical outcome are underpinned by an assumption that may be fundamentally flawed. It cannot be assumed that there is consistency of biologic structure and function between all cells in the primary tumor and those in the metastasis. This problem often is overlooked, and there is a considerable requirement for detailed comparative studies of the biology of primary tumors and their metastases.

ARTIFACTUAL HETEROGENEITY

Heterogeneity may be perceived where it does not exist because of an experimental artifact. In immunohistochemical studies, variations in staining sensitivity, antibody specificity, and preparation artifacts are common. Fixation is a particular problem, and it is surprisingly uncommon for studies on an archival series to be supported by parallel studies on freshly obtained material to control for fixation artifact and sample degradation with time. Exhaustive controls appropriate to the experimental technique are essential. Care also must be taken to account for interinstitutional variation when comparing data (14).

The failure to detect key biomarkers in significant proportions of cells is common. This raises the question of whether observers are reporting true or artifactual heterogeneity. For example, the expression of the biomarker may be below the levels of detection of the assay in use. Key regulatory molecules often are rapidly synthesized and degraded in cells. Failure to detect a target molecule or process in a static assay may give a misleading indication of the significance of the marker. An outstanding example of this problem is provided by apoptosis, which proceeds so rapidly in tissues that it completely evaded recognition until the 1970s (15–17).

HETEROGENEITY AND THERAPY

All human cell lineages possess subtle, complex, and reliable systems to guarantee the fidelity of reproduction, function, and survival over evolutionary time scales. They possess considerable redundancy in their molecular engineering to guarantee against environmental damage. This makes cell lineages extraordinarily resilient to deliberate damage by medical intervention. Over eons, cells have adapted to lifelong background and cosmic radiation, which in turn has prepared their damage repair mechanisms for modern drugs and radiotherapy.

Structural Heterogeneity and Resistance to Therapy

The anatomic heterogeneity of tumors creates natural resistance mechanisms to adjuvant therapy. Most solid tumors proliferate at the periphery, leaving the center or core relatively undervascularized. As tumors enlarge, internal blood flow and the diffusion of gases and nutrients become less and less uniform. This prevents the even perfusion of drugs throughout the substance of the tumor. The resulting tissue hypoxia, acidity, and raised interstitial pressure can be shown to impair drug uptake and biologic activity, thus increasing resistance to both chemotherapy and radiotherapy (18–20).

Heterogeneity of Damage Resistance Mechanisms

Early success in chemotherapy often is followed by relapse and subsequent treatment failure. Mutant, drug-resistant cell lines may be selected during the course of

treatment and may supersede the drug-sensitive cells. Heterogeneity of a variety of cell functions contributes to the evolution of resistance to therapy. Genetic heterogeneity produces phenotypic changes in protein and enzyme expression and function that affect the uptake and response to cytotoxic drugs or to DNA damage. Resistance to treatment is conferred de facto by the existence of a genetically diverse and pluripotential cell population. Competition for survival within the complex environment of a single tumor may be intense. As some cells are destroyed by therapy or necrosis, the biologic fitness of other cells may manifest as changes in proliferation, drug resistance, greater capacity for DNA repair, reduced rates of apoptosis, or a greater capacity to metastasize. Thus, a natural selection process is at work within the growing tumor among multipotential cells, and environmental forces such as cytotoxic therapy will effect different selection pressures on different clones.

DNA maintenance and repair (21,22) are important and heterogeneous functions of cells. DNA damage may arise spontaneously in the course of normal molecular processes, from natural or therapeutic radiation, or through chemotherapy. DNA repair imparts reproductive continuity and fidelity upon cells and may protect against a much higher incidence of cancer in the natural world. To put this capability in context, it has been estimated that the DNA of each cell, comprising 4×10^9 bases, temporarily loses 10,000 nucleotide bases each day from spontaneous DNA damage alone. There is a predicted efficiency of only three mistakes per 3 billion base pairs during DNA copying, in each of the 10^{14} cells in the human body, and in as many as 10^{16} cell generations per human life span in stem cell lines. This DNA repair capability highlights a profound problem in cancer therapy because such an evolutionary stable biologic process offers considerable compensation for DNA damage induced by clinical therapeutic maneuvers (23–26).

Heterogeneity of detoxification mechanisms (27) and of expression of the multidrug-resistant, membrane-bound, ATP-dependent glycoprotein phenotype (28–33) confer plasticity of drug resistance on tissues and tumors. Heterogeneity of p170 glycoprotein expression imparts flexibility to the capacity for response of the tumor cells to therapy.

Radiotherapy and Heterogeneity

Tumor radiosensitivity is a heterogeneous function of the proliferative behavior and the cell cycle state (34,35). Quiescent, noncycling cells may be less sensitive to radiation damage. Tissue hypoxia such as is commonly found in the center regions of solid tumors imparts radioresistance, as do intracellular damage limitation processes, such as the presence of thiol compounds to mop up superoxide radicals, and the activity of DNA repair mechanisms.

Paradoxically, both radiotherapy and chemotherapy may induce additional resistance during treatment. Cell populations respond to radiotherapy with the processes of repair, redistribution, reoxygenation, and repopulation. Cells tolerate and overcome sublethal radiation doses to varying degrees as a result of the complex interactions of the many heterogeneous processes, and resistant clones commonly come to be selected during treatment. Treatment also induces sublethal genetic damage in tumor cells, thus speeding up natural selection and increasing the risk of new mutations and genetic disorder in the tumor under treatment.

THE TOOLS TO STUDY CELL AND TISSUE HETEROGENEITY

Direct Observation, Histology, and Histochemistry

The human eye and brain form by far the most powerful image processor for rapid pattern recognition and interpretation, which are the key skills of pathologists. The conventionally stained histologic section contains an immense amount of information about tissue architecture, constitution, cell size, and characteristics. Histochemical staining aids the identification and discrimination of markers of interest and allows a degree of quantitation, either by the human observer or by automated image analysis. Qualitative assessment of pattern heterogeneity underpins the classification of tumors by grade and differentiation, and such qualitative measures may be extended to other biologic features. The observer is less efficient at quantitative tasks because fatigue and time impose severe constraints on the manual counting of cells. Human observers also are limited to observations in the visual wavelengths.

The microscope has yet to be superseded as the mainstay of pathology. However, computers and optical and laser technologies have substantially extended the range and capabilities of instruments for quantitative cytopathology in recent years. Instruments that are likely to be adopted in pathology practice in the study of heterogeneity in tissue samples must have specific characteristics. They should have a quantitative capability, rapid data acquisition and analysis, and must perform outside the range of the human eye, for example in relation to resolution or provide more information than the visible spectrum. They must present data in a simple and intelligible form that integrates with conventional pathology skills, and the data so generated must be useful to the clinical

management of disease or add to the clinical research knowledge base.

Analytic cytology mandates consistent detection and interpretation of heterogeneity of cell form and structure, for example in cervical and breast tumor screening. Automated image cytometry harnesses direct visualization and digital manipulation of images of selected cells, but the numbers of cells that can practically be studied limits its utility relative to the capabilities of the trained observer.

Fluorescence-based techniques offer great advantages in pathologic research. Fluorescence events can be measured rapidly (milliseconds) and quantitatively over a broad light spectrum (36). Many can be used to identify biologic processes and moieties either directly or when bound to monoclonal antibodies. Techniques include conventional fluorescence microscopes and confocal microscopes to study subcellular structure. Flow cytometry and laser scanning cytometry allow the study of large numbers of cells in biologic samples. Flow cytometry has been widely used in clinical pathology for DNA ploidy measurements, proliferation measurements, oncoprotein assays, and the evaluation of drug resistance (37). The principle of flow cytometry is to stream particles (cells, nuclei, or chromosomes) in suspension through one or more beams of coherent light of defined excitation wavelength. As many as six parameters can be analyzed in large numbers of particles, typically 5000 to 10,000, at data collection rates as great as 300 particles per second. Computer processing of list file data facilitates additional information analysis and clear presentation. Once they have passed through the laser beam and the flow cell, however, cells cannot be recalled, visualized, and analyzed again individually.

A number of practical constraints limit the utility of flow cytometry. One is the requirement to disaggregate tissues into fluidie suspensions for analysis. Cells can be damaged in the disaggregation process. The interpretation of flow cytometry data also requires considerable expertise and can be confounded by artifact. The characterization of cells is indirect, by a combination of cell size and light scattering features. This is acceptable in hematologic studies, for which leukocytes have precise and reproducible physical characteristics, but is much less useful in the study of cells derived from tissues and tumors.

The laser scanning cytometer is a new tool that may assist studies of tumor heterogeneity. It combines the functions of flow and image cytometry with conventional microscopic study (38,39). It shares common processes with flow cytometry in tumor phenotyping and DNA analysis (40–44) but has key advantages, in that the laser beam is scanned over the surface of the sample on a microscope slide. The optical system is a conventional microscope. The stage contains a computer-controlled stepper motor that allows precise calculation and recall of each cell position on the sample slide. The laser scanning cytometer makes approximately 100 measurements on each cell at cell densities of 100 to 1000 cells per mm^2. One thousand cells per minute can be analyzed. The microscope also can be used in conventional or dark-field modes, thus providing a powerful tool for conventional cytopathologic study.

The position (X-Y coordinates) of the cell or particle on the microscope slide is a measurement property of the machine, allowing recall, or visual or camera inspection of selected populations of cells, and the reevaluation and repositioning of cells after additional staining. A charge coupled device (CCD) camera attached to the microscope allows digitization, display, manipulation, and recording of images. Thus, individual cells and rare events can be identified within complex cell populations, visualized, and analyzed directly. This allows confirmation of the identity of cells within a complex, heterogeneous population, and the direct exclusion of artifact. This is a major advance in quantitative analytic cytology.

Mathematics

There are no satisfactory quantitative or mathematical models or bench marks to predict, describe, or analyze heterogeneity in tumor histologic study. For example, in the histologic study of a biomarker in fixed or fresh tumor material, it is not certain how many tissue blocks should be studied from any one tumor to ensure true representation of that marker. Within each tissue block, how many sections should be studied and how many microscope fields within each section? Will the parameters vary between an anaplastic tumor with little visible variation and a well differentiated, heterogeneous tumor?

Heterogeneity may be truly random in its development and manifestations. Conversely, it may be a mathematically predictable expression of biologic order. A novel proposal is that chaos theory may help to explain tumor heterogeneity (45). Chaos theory is the mathematics of the seemingly complex but nonrandom and highly ordered behavior that may be shown by simple, nonlinear systems. Thus, chaotic (ie, predetermined) patterns may exist in the evolution of the genotype and phenotype in individual tumors and in biologic populations. If so, their identification may mark simple disturbances in the underlying molecular processes. Computational techniques have not yet evolved to allow the detection and prediction of chaos in complex biologic systems such as tumors.

Mathematical models or protocols that imposed on researchers a consistent approach to recording the heterogeneity inherent in the expression of any marker under study in any one locus of a tumor would be valuable. The absence of consistent protocols should not deter observers from attempts to address the problem of heterogeneity in the methodology of measurement and the discussion in each and every clinicopathologic series.

CONCLUSIONS

Biologic heterogeneity is the key to success and survival in the natural world. Heterogeneity of individual genotype is the foundation of Darwinian evolutionary competition and of species diversity. Thus, it is unfortunate that it also is a key feature of malignant change. Heterogeneity of tumor biology poses major problems for researchers in anatomic pathology. Failure to pay due regard to the problem of heterogeneity devalues many clinical research studies and complicates their interpretation.

We need to recognize the problems posed by heterogeneity in tumor biology and to embark on a systematic study of those problems. We need to develop standard, quantitative indices of heterogeneity in cell and tissue pathology and to raise the profile and standard of reporting of heterogeneity in all scientific papers.

The intellectual framework and the tools are in place to allow pathologists to move from descriptive reporting to a more critical appraisal of the complex patterns and messages encrypted in the cell sample and tissue section. The next century promises to be a fascinating era in which human intelligence and technology confront the fundamental complexities of corrupted biology.

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