Tissue Heterogeneity as a Pre-analytical Source of Variability

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Contents

A low level of reproducibility is the shortcoming of clinical studies where human tissues are used, especially in oncology [1, 2]. This could be due to the high variability of the pre-analytical conditions of tissue managing and preservation, but also to other two causes. One is the low level of standardization of the methods that is pertinent to the analytical phase. Heterogeneity of tissues is the other problem and it can be considered to be actually related to pre-analytical procedures. Indeed, it is a pre-condition that has to be taken into consideration before the specific analysis. Not all the consequences of heterogeneity can be more or less easily avoided by carefully choosing the tissues to be analyzed. Some types of heterogeneity are strictly related to the complexity of the carcinogenesis phenomena and are not easy to localize with a proper micro-dissection. It is anyway possible to improve reproducibility of tissue molecular analysis by taking into consideration at least some of the aspects of tissue heterogeneity. It is also important to recognize that

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different types of molecular analyses can be differently affected. Gene expression analysis is even more perturbed than DNA sequencing by which only different genetic cell populations can be recognized in the same sample.

1 Different Types of Heterogeneity

Different types of heterogeneity have to be considered when analyzing human cancer tissues for diagnostic or clinical research purposes. We have to consider macroscopic, microscopic, and molecular types of variation. Macroscopic heterogeneity is the clinical variation in patients, such as type of tumor and treatment, or patients' age and gender. This type of clinical heterogeneity is strictly related to the design of the study and, if properly driven, it does not affect the result reproducibility of a study because these characteristics have been chosen and well known from the beginning of the research. In most cases, this clinical variability is the reason why the study was performed and is not considered in this paper.

Other types of heterogeneity are more insidious, like heterogeneity in cancer tissues or the more recently detected molecular heterogeneity related to clonal evolution or autocrine, paracrine cell interaction (Box 1). These types of heterogeneity can heavily affect the results of molecular analyses at the clinical and research level and should be considered as the most important cause for the scarce reproducibility of clinical research. On the other hand tissue related heterogeneity is well recognized but very often under-evaluated as a source of analytical variability, especially in clinical research but sometimes also at the diagnostic level and it sure can be improved. Molecular heterogeneity is more complex and still in a research phase. At the moment we do not have sufficient information to manage the problem properly.

Heterogeneity in cancer tissues is one of the characteristics that suggest a multidisciplinary approach. Clinical heterogeneity must be evaluated mostly by oncologists, tissue related heterogeneity can only be tackled by an experienced pathologist and molecular heterogeneity is still at the center of a research process and must be evaluated by experts in molecular biology, oncology, and pathology.

Box 1: Different Types of Heterogeneity Affecting Diagnosis or Clinical Research

Clinical Heterogeneity related to different patients' conditions (different tumor type, ethnicity, age, therapy, etc.)

Tissue Related Heterogeneity

- Related to tissue complexity (fibrosis, inflammation, necrosis, normal residual tissues…)
- Related to histological heterogeneity (different differentiation pattern of the same tumor)
- Different functional areas (border vs center of the tumor)

Molecular Heterogeneity

- Genetic clonal evolution
- Epigenetic clonal evolution
- Phenotypic plasticity
- Heterotypic interaction

1.1 Tissue-Related Heterogeneity

Tissue-related heterogeneity is a well-known pattern that is always detectable in cancer tissues at a more or less high degree. Sometimes this variability is of very low level and does not affect molecular analysis, but sometimes it can give highly contradictory results depending on the analyzed area. It is possible to recognize at least three types of this kind of heterogeneity. The first one is related to *tissue complexity*. Tumor tissues do not only contain cancer cells but a variable degree of fibrosis that is characteristic of the tumor type as a desmoplastic reaction. It could also be related to the size of the tumor with central necrosis substituted by a dense fibrosis due to insufficient neo-angiogenesis and related hypoxia. In most tumors there are also cells with normal genome, such as reactive inflammatory cells that are part of the carcinogenetic process, or residues of normal tissues involved in the invasion process. It is easy to realize how the presence of one or more of these cancer tissue components, especially if they are quantitatively relevant, can completely alter the results of molecular analysis, giving false positive or negative results. This is usually the only tissue heterogeneity taken today into account during tissue micro-dissection, when histological examination is performed and mechanical or laser dissection is suggested. In many tumors such as breast and colon cancer, it is common to find normal tissue together with the neoplasia in histological specimens and this mixture is often accepted in the sample analyzed for diagnostic molecular signatures such as Mammaprint, Oncotype DX, PAM50. It has been shown that the presence of normal tissue can modify, even if in a limited quantity, the category of risk to a less aggressive than the one detected in the pure breast tumor tissue [3].

A more complicated type of heterogeneity is the one related to *different histological patterns* in the same tumor, which sometimes could also represent different levels of molecular differentiation. At the moment this phenomenon is still badly defined and deserves more attention by pathologists and molecular biologists. For example, we could expect to find differences when analyzing differentiated and anaplastic areas of the same tumor that can often be found in human cancers. We need to better define those characteristics not only in a general way but also for

specific types of tumors, by evaluating the importance of this factor in the reproducibility of molecular analysis in diagnostics and clinical research.

For quite a long time we have been aware that there are *different functional areas* in a tumor such as borders and the center of an invasive neoplasia that display different gene expression patterns [4]. Usually the central part of the tumor is more affected by hypoxia and cellularity is low with a higher fibrotic component, whereas the border can be crowded with cells with activated proteolytic enzymes and a more frequent interaction with reactive cells. The border itself can give better information on the aggressiveness of the tumor. When the tumor is large and the position of the analyzed tissues is unknown, this can heavily affect molecular diagnostics and research analyses.

Of course there are differences in tissue heterogeneity among tumour types and a more complete analysis is necessary to establish specific characteristics. However it is possible to consider some general rules that could help a higher level of standardization like those reported in Box 2.

Very short time between cutting micro-dissected area sections and the extraction of nucleic acids (especially for RNA) should pass, otherwise this could be another pre-analytical source of variability due to possible further degradation of nucleic acids by contamination of environment nucleases.

Box 2: Suggestions for a Practical Approach to Tackle Tissue Heterogeneity

Small biopsies

1. Histological evaluation of tissues
2. When possible micro-dissection (including border of the tumor and avoiding stroma, normal t. residues)
3. Digital record of the selected tissues

Surgical specimens

1. Histological evaluation of the tissues with topographical definition (identification of the infiltrative border)
2. Micro-dissection: single or multiple sampling, depending on the type of lesion and the histological pattern:
   a. in single sample, this should be taken from the infiltrative border, with a minimal stromal component
   b. for multiple sampling the topographical location of the micro-dissected areas should be recorded.
3. Digital record of the micro-dissected areas as integral part of result evaluation (specific IHC could help a morphometric cancer cells versus stroma evaluation)

1.2 Molecular Heterogeneity

Molecular heterogeneity in cancer is related to cancer progression. It is well known that phenomena like microsatellite instability or chromosomal instability occur in many types of tumors. Accumulation of genetic errors of sequence in DNA or gene copy number alterations are always present in cancer. These alterations are not uniform in the tumors and sometimes they are present in localized cell clones. Molecular heterogeneity is not only related to structural damages of DNA. Silencing of gene expression by promoter methylation is also on the basis of tumor progression and these phenomena are also clonal. So we have to consider a genetic and an epigenetic clonal evolution that can affect only part of the tumor and sometimes very few cells.

More unpredictable and difficult to detect is the so called phenotypic plasticity. This is related to autocrine-paracrine phenomena between cancer cells that show the same genotype but with evident different patterns of functional gene expression.

Another type of phenotypic plasticity seems to be stochastic, for which the level of gene expression and the time of expression can vary from cell to cell according to efficiency of the single cell machine.

Of note is also the heterotypic interaction between cancer cell and stromal component cells such as lymphocytes, macrophages, fibroblasts, etc., for which cytokines can stimulate tumor progression. Phenotypic plasticity and heterotopic interaction are strictly related and sometimes undistinguishable mechanisms and they will be discussed together.

2 Genetic Clonal Evolution

Genetic clonal evolution in human tumors is strictly related to genomic instability in cancer. Instability can give different alterations in different cells and the clones with growth advantages tend to expand. These alterations can be common to all the tumor cells or are restricted to same cells only. This process was presented as a Darwinian phylogenetic evolution in cancer [5].

It is well known that there are clonal genotypes with expansion or decline of clonal populations over time. From these clones, rare or frequent in the tumor, the metastatic process can originate. Some of the clones derived from random genetic drift can also show a neutral relationship without discernible phenotypic consequences [6]. There are also tumors that do not present an evident clonal structure based on genome aberrations (Box 3).

Box 3: Molecular Heterogeneity and Genetic Clonal Evolution in Cancer [6]
Existence of different clonal genotypes in the same tumor
Expansion and decline of clonal populations over time
Existence of internal spatial variation in tumor composition
Emergence of drug-resistant malignant cells
Metastatic cells from sub-clones (rare or common)
Absence of clonal structure based on genome aberrations in some cancers
Existence of neutral clonal relationships (from random genetic drift without discernible phenotypic consequences).

One of the most relevant consequence of genetic clonal evolution is the evidence that it is related to acquired resistance to new target biological treatments of cancer. It was shown that after anti-EGFR therapy in metastatic colon cancer the majority of the cases that were KRAS wild turned out to be mutated if detected after some time in circulating tumor cells. On the other hand the cases treated with traditional chemotherapy only continued to be KRAS wild type [7]. This can be related to the expansion of mutated minor clones driven by the specific therapy.

3 Epigenetic Clonal Evolution

For epigenetic clonal evolution in cancer the considerations to be made can be similar to those for genetic clonal evolution. It is well known that silencing of tumor suppressor genes can be due to promoter methylation and this process is common to most of the tumor types. Also some mechanisms are known in tumors that highly increase the frequency of hyper-methylation of gene promoters. The so called CpG island methylator phenotype (CIMP) was first recognized in the hyperplastic polyps of the colon as a new type of carcinogenesis process [8]. Hypermethylation of the CpG-island promoter of tumor-suppressor genes and of miR genes inactivates transcription and the epigenetic changes are inheritable, for this reason they are strictly part of clonal evolution of cancer. It is important anyway to underline that not hypermathylation but hypomethylation of DNA is a landmark of malignant cells and this can reactivate intragenomic endoparasitic DNA repeats (L1 and Alu). These undermethylated transposons can be transcribed or translocated to other genomic regions with the promotion of chromosomal rearrangements.

Also other epigenetic mechanisms like deacetilation or methylation of histones can silence tumor-suppressor genes even without hypermethylation of the promoter CpG islands (Box 4).

Box 4: Molecular Heterogeneity and Epigenetic Clonal Evolution
Epigenetic changes are inheritable and part of clonal evolution
It is possible to modify epigenetic alterations
Hypomethylation of DNA in malignant cells can reactivate intragenomic endoparasitic DNA repeats (L1 and Alu). These undermethylated transposons can be transcribed or translocated to other genomic regions with chromosomal rearrangements.
Hypermethylation of the CpG-island promoter of tumor-suppressor genes and of miR genes inactivates transcription
Deacetilation or methylation modification of histones can silence tumor-suppressor–genes with or without hypermethylation of the promoter CpG island.

4 Phenotypic Plasticity and Heterotypic Interaction

A specific tumor genotype can express a wide range of phenotypic manifestations, called phenotypic plasticity, in response mostly to the surrounding microenvironment stimulations. These are the result of autocrine and paracrine mechanisms related to the same tumor cells or to reactive normal cells. In this latest case it is called heterotypic interaction. In most of the cases the two mechanisms are almost undistinguishable, and very often tumor cell autocrine activity cooperates through the heterotypic interaction with the reactive cells, taking to very complex functional relationships [9, 10]. Similar examples are very well known. Phenotypic plasticity is frequent as an adaptation of the cell to common aspects related to cancer, such as hypoxia, which is related to tumor proliferation and aggressiveness. There is also cooperation between hypoxic cells and non hypoxic ones in the same tumor because the lactate produced by hypoxia can be transformed into pyruvate and utilized by the non hypoxic areas [11, 12]. In breast cancer, hypoxia induces lymphangiogenesis around the tumor tissues, facilitating the lymphatic way of diffusion of the cancer cells [13].

Also “stemness” is related to autocrine and paracrine phenomena [14, 15] and the same epithelial-mesenchymal transition (EMT). These are facts that show how progression of the tumor is related not only to genetic and epigenetic clonal mechanisms but also to different functional phenotypes that can coexist in tumor cells with the same genotype. This is true not only for coding genes but there is increasing and wide evidence also for non-coding RNAs like microRNA expression [16, 17].

There is another type of phenotypic plasticity related to the different efficiency of the cell machines, especially those of transcription and translation that appear to be stochastic and different from cell to cell of the same tumor [18]. It was shown experimentally in cell lines that different cells with the same genotype have different

time for programmed death when the process of apoptosis is stimulated simultaneously [19]. Pathologists have known this phenomenon for a very long time. Indeed, in immunohistochemistry the positivity for any antigen hardly ever appears to be uniform in the same type of cells, but it varies a lot in intensity. This variation is taken as a criterium to evaluate specificity of a new antibody.

An example of cell machine efficiency is the fact that chaperon proteins can modulate the effect of a mutation at the expression level and the result is again referred as stochastic with often a very wide range of overlapping phenotypes between mutated and wild cells [20]. Tyrosine kinase receptors are client of a chaperon protein HSP90 and the efficiency of HSP90 modulates the cellular response of these receptors. This could also be the reason why different tumors or sub-clones of a tumor can react differently to the receptor inhibition in cancer treatment. For this reason inhibition of HSP90 was proposed as a developing cancer treatment [21].

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