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Review Article

Pre-analytics and tumor heterogeneity

Serena Bonin*, Giorgio Stanta

Department of Medical Sciences, University of Trieste, Trieste, Italy

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ABSTRACT

When dealing with pre-analytics for tissues, it is often that case that tissue heterogeneity, and particularly tumor heterogeneity, is not taken into account as a preliminary condition for obtaining reproducible results in molecular analysis at the diagnostics and clinical research levels. It is well known that when sampling tumor tissues in different areas, for example the border or the central area of the tumor, different genes are expressed and, due to polyclonality in most tumors, different areas can have different DNA and epigenetic alterations. For this reason, it is extremely important to establish and standardize specific tissue sampling protocols for molecular extraction as well as *in situ* molecular methods. A correct approach to heterogeneity is the basis for a more reproducible and exchangeable type of molecular analysis that can provide useful information at the prognostic and predictive levels. Heterogeneity should also be taken into consideration during cancer treatment, since therapy modifies the clonal composition of tumors. Here, the different types of tumor heterogeneity and the improper pre-analytical conditions in tissue processing that can generate heterogeneous artefacts are described.

Introduction

Tumor progression is mostly based on clonal evolution, which in cancer is strictly related to aggressiveness and to primary and acquired resistance to therapy. This evolution is studied by analyzing intra-tumor heterogeneity at the spatial and temporal levels. Therefore, it is important to recognize and distinguish true from false heterogeneities. False heterogeneities can be related to inappropriate pre-analytical conditions. Separation of clonal heterogeneities from those coming from micro-environmental interactions is mandatory for sound analyses. These analyses are nowadays even more important because of their necessity not only for research, but also and specifically for diagnostic activity [1]. This is related to the fact that the range of variation at the level of inter-tumor heterogeneity often exceeds the limits defined by any molecular classification [2,3]. Reproducibility of analyses is essential and fundamental for any clinical or research activity.

Intra-tumor molecular heterogeneity of DNA sequence alterations may be the result of clonal evolution, but can also be the consequence of the tissue fixation process as well as phenotypical heterogeneity, which at the level of RNA can be related to a number of technical problems, such as impaired reverse transcription in cDNA synthesis. However, before starting to analyze the possible sources of false heterogeneity it is essential to inspect the possible patterns of true heterogeneity [4–6].

Practical approach to heterogeneity

Heterogeneous molecular patterns can be detected as biomarkers at the genetic or expression levels using extraction methods or maintaining tissue morphology through the use of *in situ* techniques. Such analyses can be applied at 1) the spatial level within a primary tumor, 2) the temporal level by the analysis of metastatic tissues, and 3) the type of sample using tissue or liquid biopsies. In all cases, reproducibility of the analytical results is the prerequisite for any clinical decision. It is also important when sharing clinical records between hospitals and health institutions.

Histologically, there is already wide experience of morphological heterogeneity: in the same tumor it is quite common to find different histological patterns or at least a different level of cellular differentiation. Those histological differences are also often related to differences in the detection of biomarkers at a molecular level [7]. Molecular differences have been recognized in different tumor areas, such as between the central part and the border of the tumor [8] or between primary tumors and metastatic lymph nodes [9,10].

Abbreviations: CNNA, copy number alterations; EMT, epithelial-mesenchymal transition; FFPE, formalin-fixed and paraffin-embedded; IHC, immunohistochemistry; ISH, in situ hybridization; ITH, intra-tumor heterogeneity; NGS, next generation sequencing; RNA-seq, RNA-sequencing; SNV, single nucleotide variants; UNG, uracil N-glycosylase

* Corresponding author at: DSM-Department of Medical Sciences, Cattinara Hospital, Strada di Fiume 447, 34149 Trieste, Italy.
E-mail address: sbonin@units.it (S. Bonin).

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There are several types of intra-tumor molecular heterogeneities: some are clonal while others are merely functional at a translational level. DNA sequence alterations are clonal and related to tumor genomic instability. In this group of alterations, it is important to discriminate between mutations of a single or a few nucleotides from copy number alterations (CNA) of large sequences, as they have a recognized different clinical meaning in many tumors [11–18]. A high number of mutations is frequently related to tumor lymphocyte infiltration and a higher level of neo-antigens [16]. Although influenced by the micro-environment, epigenetic alterations at the gene and histone levels can also be considered as clonal. Methylation of gene promoters is a basic mechanism of tumor progression as well as histone methylation and deacetylation. These mechanisms are frequently detected in tumors with direct influence on gene expression [19].

The interaction among different clones, expressing different translational patterns, and the micro-environment can define a complex functional pattern, which may be heterogeneous in different areas of the tumor. Phenomena such as epithelial-mesenchymal transition (EMT) or stemness can be highly heterogeneous. This type of heterogeneity is not clonal, but translational and is referred to as 'functional plasticity' [5].

In routine pathology it is common to find immunohistochemical positivity of a biomarker varying among the same types of cells. This phenomenon, which is also observed in clonal cells in a culture plate, is related to different efficiencies in transcription, translation and chaperone protein levels. This type of molecular heterogeneity, mostly unrelated with the others, is termed stochastic plasticity [20] and has been proposed as being one of the causes of chemotherapy resistance [21]. Stochastic heterogeneity must be carefully considered when dealing with studies investigating heterogeneity at the single cell level. All the described intra-tumor heterogeneities can then be functionally related to the interactions between tumor cells, with the stromal micro-environment and with the clinical treatment. The complex pattern deriving from those interactions is summarized in Fig. 1.

Heterogeneity as a whole should be considered as one of the major sources of the irreproducibility in molecular analyses. Together with poor pre-analytical conditions and the lack of standardization in analytical methods, it represents the basis of the lack of reproducibility in clinical research that is currently a highly debated issue [22,23]. To evaluate heterogeneity, it is important to consider a new type of tumor tissue sampling with a standardized analysis. In the European Society of Pathology and in the Organization of European Cancer Institutes, some groups studying specific tumors have already started to test novel types of sampling for tumors over two cm diameter [24].

Pre-analytical conditions affecting tumor heterogeneity

The pre-analytical conditions in biological material are a major issue to be resolved in obtaining reproducible and exchangeable molecular diagnostics and clinical research results. Overall, it has been estimated that around 1/3rd of the irreproducibility can be related to pre-analytical conditions [25]. This topic has been addressed by several European organizations and industries in large European projects, such as SPIDIA and today SPIDIA4P (https://www.spidia.eu/). Many pre-analytical conditions can affect tissue and tumor heterogeneity (See Fig. 2) producing a false biological heterogeneity that can be misleading in research and in diagnostics. Here we evaluate stepwise the variables associated with tissue processing that can be a possible source of false heterogeneity.

Warm and cold ischemia

Warm ischemia refers to the tissue prior to its removal from the body after the blood supply has been stopped. Blood vessel clamping during surgical procedures causes hypoxia, ischemia and metabolic stress [26]. Warm ischemia time can be extremely variable due to: 1) the type of surgery or intervention, 2) the different surgical procedures, 3) the particular patient conditions and 4) the surgeon's skill. Such variable conditions can affect the detection of clinical biomarkers and apparently may increase tumor heterogeneity. Warm ischemia-induced metabolic activity in living cells during surgical intervention can induce alterations in survival or apoptotic processes, which can be detected as changes in transcript levels of some genes [27]. Surgical manipulation has already been reported to cause significant gene expression changes at the mRNA level [28,29]. Intra-tumor molecular heterogeneity with gene expression variation has been reported in separate areas of the same tumors and it has been discussed that it could be related to intra-tumor sub-clonal diversity producing a cell-dependent response to warm ischemia [27]. Surgical procedures can be other sources of false heterogeneity. In the operating theatre, the use of specific techniques (*e.g.* heat cutting) or the mechanical traction and distortion of tissues can influence not only gene expression, but also protein coagulation or protein diffusion into extracellular spaces, with possible consequences for immunohistochemistry (IHC) results. Reliability in protein detection and RNA analysis can also be affected by common pathological processes that can be enhanced during surgical procedures, such as hemorrhagic diffusion in tissues, necrosis, inflammation or apoptosis.

In biospecimen science, cold ischemia time is defined as the time interval between removal of the sample from the body until further preservation, including chemical fixation or snap freezing [30]. Cold ischemia time can also be highly variable depending on the hospital structure and organization. If the operating theatre is close to the

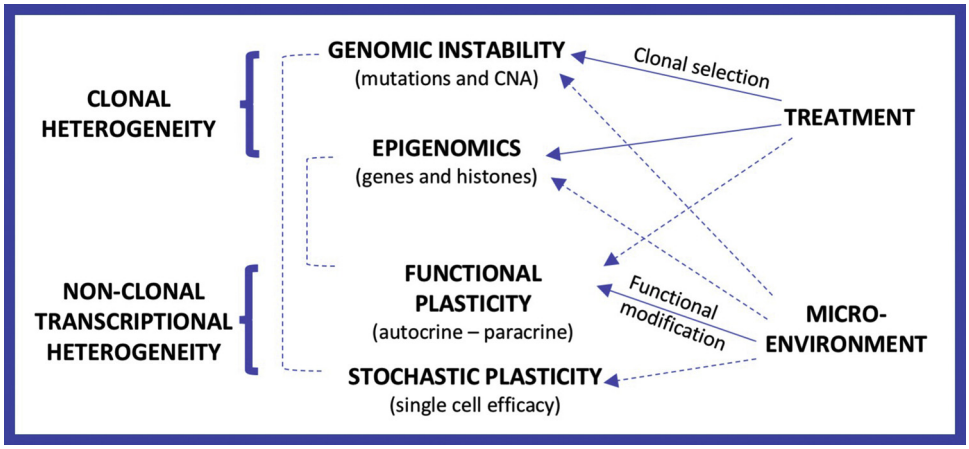

Fig. 1. Graphical representation of the complex interactions among different types of heterogeneity.

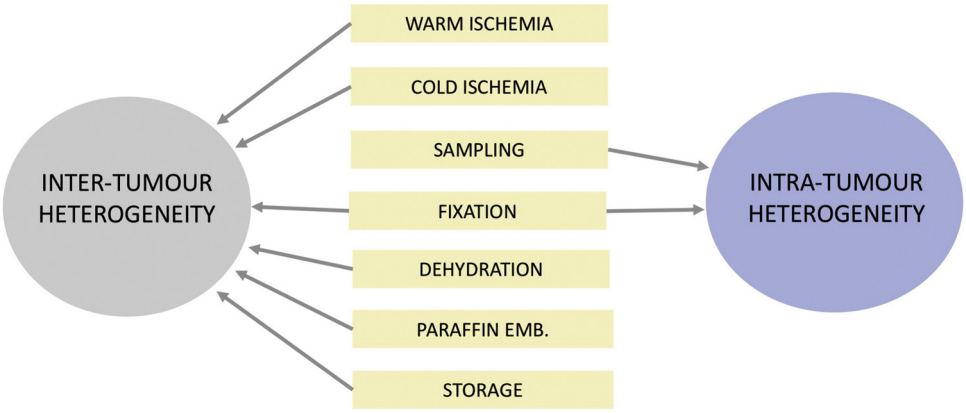

Fig. 2. Pre-analytical variables potentially involved in false tumor heterogeneity.

pathology department, the specimen can be transported fresh in a short time at room temperature or in wet ice to the pathology lab. In that case, false heterogeneity of tissues can derive only from air drying of the external part of the specimen with serious consequences for immunohistochemical examination. If the ischemia time is short and less than 30 min, the preservation of macromolecules is very good. Longer times can occur where tissues specimens are only sent to the pathology department at the end of the day or where tissues are delivered from another hospital and formalin is used. Transport in formalin should be avoided, and not only for biosafety concerns. However, some hospitals still store specimens in formalin over weekends before transport. Transport in formalin can have important implications in creating false tissue heterogeneity at the molecular level, in particular for large specimens before grossing, since the degree of fixation can only be superficial, leaving the internal areas to be different [31,32]. This can lead to external over-fixation while the inner portion may be damaged by prolonged hypoxia. Such events can be related to modification of phosphoproteins [33]. The transport of surgical specimens in plastic bags under vacuum at 4 °C represents a possible solution [34]. Macromolecules are well preserved for at least three days and tissues can also be used for biobanking and primary cell culture [34,35].

Warm and cold ischemic times may affect gene expression differently. Gene expression changes seem to occur early in normal and tumor tissue during surgery with a gradual increment over time [29,36]; however tumor tissues seem to have higher variability and reactions to stress and hypoxia [30]. The effects of warm and cold ischemia have been classified as ischemia-induced metabolic responses which are early events after reduction of the blood supply, and ischemia-induced degradation on a cellular and tissue level as a result of hypoxia and stress [30]. Cold ischemia time has also been related to RNA integrity with variable effects depending on the tissue type [27]. During prolonged ischemic time, some affected transcripts may give a higher or lower expression level. Genes involved in metabolism, mRNA or protein processing, and in cell cycle regulation usually show a lower expression because of the response to ischemic stress or transcript loss due to mRNA degradation [37]. Storing tissue samples at 4 °C could reduce the metabolic rate, reducing the variation in gene expression [27].

Alterations in transcript expression levels are also related to cytokines, hypoxia and metabolic acidosis that can cause activation of transcription factors and modification in signaling pathways [38]. Thus, increased transcription levels of stress response genes, such as those sensitive to hypoxia, can be the result of hypoxic stress during ischemia and could confound the assessment of prognostic gene-expression based biomarkers [37]. The pattern can be even more complex as different antigens have been reported to have different reactions to over-fixation or hypoxia [39].

Sampling

Even today there is no standardized sampling procedure dealing with intra-tumor heterogeneity. Total tumor sampling is the ideal and possible for small tumors. The problem for pathologists is mostly related to large tumors, which are far from being totally analyzed [40]. Some initial proposals have been made and initially tested in certain European organizations [24]. Here, the response of biomarkers depending on the tumor areas was investigated and randomly analyzed. This picture of the biomarker distribution can actually be related to a true intra-tumor heterogeneity. Other solutions of multi-site tumor sampling have also been recently reported [40,41]. False molecular heterogeneity could also derive from common pathology procedures referred to, such as delayed tissue transport in formalin over a weekend (see above).

Fixation

Although formalin fixation and paraffin embedding can have an effect on molecular studies, they appear to be settled procedures. However, in daily pathology practice, this is not completely the case and molecular procedures should investigate formalin-fixed and paraffin-embedded (FFPE) materials and their possible pitfalls. It is well known that formalin fixation leads to the formation of adducts in nucleic acids and proteins and crosslinks between them [42] as well as that among the nucleic acid bases adenine is the one most modified, with nearly 40% of mono-methylol adducts [43]. Thus, different fixation and pre-fixation time can alter protein as well as nucleic acid integrity and detectability. When different fixation times are applied in tissues from primary tumors and metastases, it is not surprising that the detection of some biomarkers can be discordant with reality as the result of different pre-analytical conditions. Both under and over-fixation may lead to false negative or positive results in IHC protein expression [44]. In particular, under-fixation has been shown to be detrimental to IHC and *in situ* hybridization (ISH) staining results [45].

Hydrolytic deamination of cytosine to uracil has been identified as a major source of sequence artefacts in DNA extracted from FFPE samples, and transitional C:G to T:A are the most frequent type of single nucleotide variants [46]. Sequence artefacts are more readily detectable when low copy numbers of FFPE DNA are tested [46] representing a possible source of false intra-tumor heterogeneity in FFPE materials. For next generation sequencing (NGS), deamination variants have been reported to increase over time of block age and formalin fixation time; this effect can be partially mitigated by uracyl N-glycosylase (UNG) treatment of DNA prior to sequencing [47]. Related allelic frequencies have been detected below the 5% reporting threshold for the NGS assay used in [47] and hence would not have been reported clinically. However, DNA changes with mutant allele frequencies of < 3%

detected in FFPE samples may be artefactual in a non-negligible fraction of cases [48]. UNG pre-treatment of DNA is mandatory to identify true changes in archival samples and to avoid misinterpretation [48]. Furthermore, suspected intra-tumor heterogeneity (ITH) events that can be less frequently represented should be verified by re-sequencing the same FFPE block for confirmation [47].

The investigation of phenotypic heterogeneity in FFPE tissue also suffers from RNA and protein analysis changes related to over-fixation. It is well known that longer fixation times in formalin are responsible of higher degradation of RNA obtained from FFPE. Disparities in fixation time and in sequence detection for the same transcript can generate confusing results in RNA expression profiles from FFPE. Several studies have evaluated the performance of FFPE specimens with high throughput assays, including gene expression microarrays, RNA-sequencing (RNA-seq), and NanoString, showing that FFPE can provide reliable data for gene expression [49]. Gene expression quantification has produced reliable results by RNA-seq, whereas gene fusion and single nucleotide variation (SNV) detection have been reported as unfeasible with FFPE specimens [49]. With respect to reverse transcription (RT)-PCR analyses in RNA from FFPE tissues, specific technical steps can be involved in false heterogeneity detection. RNA from FFPE tissues is modified by the formation of a *N*-mono-methylol ($N-CH_2OH$) adduct, which is usually followed by electrophilic attack to form a methylene bridge between amino groups [50]. This is different from the reactivity among nitrogenous bases, with adenine having the higher rate of mono-methylol addition; interruption of RT during cDNA synthesis due to the methylol addition has been shown [43]. Although several authors have reported that mono-adducts can be removed or by heating [43,51] or specific treatments [42,52] and different RT reactions have been optimized for RNA from FFPE [53], cDNA synthesis has been shown to be one of the most sensitive analytical steps which is impaired when using RNA from FFPE samples [54].

Dehydration and paraffin embedding can also alter RNA and proteins, which can give false temporal evolution heterogeneity in patient samples examined at different time periods. Incomplete dehydration can affect RNA and protein preservation in tissue storage [55]. Proteins can also be altered by the high paraffin-embedding temperature [55].

Tissue storage

Tissue storage can have some effects on the detection of biomarkers, with different impact depending on the investigated biomolecule and analytical method. This can have possible implications for tumor molecular heterogeneity when comparison is required between samples related to different temporal evolution of the pathological process. FFPE tissue storage conditions may vary. Archives may be located in damp premises, on the basement floor of laboratory buildings or at a distance with little control over storage conditions. Current knowledge about the effects of tissue block storage on preservation of antigens and nucleic acids is mostly limited to a few years and little is known about long storage periods [56]. It was shown recently that antigenicity of cytoplasmic antigens tested was maintained in FFPE tissues for 60 or more years, but reduction in immune signal was found for those antigens requiring heat-based antigen retrieval or with a membranous or nuclear location [56]. However, for RNA *in situ* hybridization, significant signal reduction has been detected after 1 year of tissue block storage at room temperature [57]. Nowadays that aspect can be overcome by acquisition of the digital images of the immuno-stains or ISH at the time of diagnosis for a further comparison. Regarding nucleic acids, degradation during storage of paraffin blocks at room temperature has already been shown both for FFPE and PaxGene fixed samples [58,59]. The possibility of preventing that degradation by cold storage is however not realistic in routine pathology [58].

Data quality in high throughput studies with FFPE tissues has been shown to be negatively correlated to storage time, with the exception of the small miRNAs which are less affected by the degradation of RNA [49].

For long term storage, it has to be considered that in the past standardization of pre-analytical conditions, especially fixation, was not a matter of discussion. Lower care over the duration of fixation was quite common. Analyzing old tissues compared with recent biological material, it is easier to encounter over-fixation damaging biological macromolecules. Another problem could be the thickness of the fixed specimens, with consequent intra-tumor false molecular heterogeneity related to outer over-fixation and inner prolonged hypoxia damage. Incomplete tissue dehydration could be a cause of protein and nucleic acid degradation during storage, together with humidity and high temperature of storerooms [55]. At the protein level, it has been shown that nuclear and membrane antigens were more sensitive to a reduction of reactivity in IHC in FFPE tissue with storage and that they need the use of retrieval techniques [39].

Conclusions

Clinical tissues samples are becoming increasingly used to define follow-up results better and to support clinical research. Therefore, it is important to recognize all possible conditions that can cause false molecular alterations in order to improve the quality of those archival tissues reducing false inter- and intra-tumor heterogeneity. In Table 1 a summary of possible pitfalls in tumor molecular heterogeneity is

Table 1
Type of intra-tumor heterogeneity and pre-analytical conditions in tissue processing.

Type of heterogeneity	Standardization	Comments
Intra-tumor heterogeneity as source of false interpretation		
Clonal evolution	Standardized multiple sampling	Not performed in the past
Functional plasticity	Standardized multiple sampling	Not performed in the past
Stochastic plasticity	Awareness in single cell expression interpretation	
Pre-analytical conditions as source of false heterogeneity		
Warm Ischemia	Information on the medical/surgical treatment	Recommended, but very often not specified in the past
	Annotation of warm ischemia time	Recommended, but very often not specified in the past. Artefactual results are possible for hypoxia-inducible gene analyses.
Cold ischemia	Fresh tissue transport	Very short time/ tissue air drying
	Formalin transport	Outer over-fixation and inner hypoxia
	Vacuum transport	Well preserved for 3 days. Recommended procedure for surgical samples.
Sampling	Standardized multiple sampling	Not performed in the past
Formalin fixation	Standardized time/sample thickness	Frequent over-fixation in the past. Quality controls needed for biomolecular analyses
Paraffin-embedding	Incomplete dehydration	Errors in comparison of samples for temporal evolution
	Paraffin temperature	
Storage	Low humidity and temperature	Errors in comparison of samples for temporal evolution. Quality controls needed for biomolecular analyses.

presented in relation to tissue processing. Nowadays, it is fundamental to acknowledge that only specifically agreed procedures are allowed in bio-specimen processing [60–62]. The availability and the use of CEN technical documents and ISO standards for pre-analytical procedures for *in vitro* diagnostics are prerequisites to having higher quality samples for both diagnostics and clinical research. When using older archive tissues, in cases where technical specifications for pre-analytics in tissue processing have not been applied, it is fundamental to determine the degradation level of the tissues by the use of specific controls to establish the quality of the macromolecules, as already proposed in the literature [54,63–65]. However, in those specimens, we should acknowledge that intra-tumor heterogeneity cannot be evaluated where multiple or total tumor sampling has not been carried out. In addition to pre-analytics, analytical procedures also need further standardization by the use of standard operating procedures (SOPs), allowing a higher level of reproducibility and diagnostics.

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