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The challenge of gene expression profiling in heterogeneous clinical samples

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ABSTRACT

Almost all samples used in tumor biology, such as tissues and bodily fluids, are heterogeneous, i.e., consist of different cell types. Evaluating the degree of heterogeneity in samples can increase our knowledge on processes such as clonal selection and metastasis. In addition, generating expression profiles from specific sub populations of cells can reveal their distinct functions. Tissue heterogeneity also poses a challenge, as it can confound the interpretation of gene expression data. This chapter will (1) give a brief overview on how heterogeneity may influence gene expression profiling data and (2) describe the methods that are currently available to assess transcriptional biomarkers in a heterogeneous cell population.

1. Introduction

The official NIH definition of a biomarker is: "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [1]. Many different uses of biomarkers fall under this broad definition, including screening (i.e., detection of pathology in the general population), monitoring (i.e., determining the disease status and evolution over time in a patient with a known illness), differential diagnosis (i.e., distinguishing one disorder from other, similar disorders) and use as prognostic and predictive factors. For this review, we will focus on prognostic and predictive biomarkers. A prognostic marker is associated with patients' outcome regardless of the administered treatment, and is thus reflective of the natural progress of the disease. A predictive marker is associated with response to a specific therapy, and can thus aid the physician in determining the optimal treatment strategy. Besides these uses with direct clinical consequences, biomarkers can also increase our knowledge of tumor biology, by using them to identify and quantify specific cell types present in a tissue or fluid, or isolating subsets of cells from complex materials which can then be characterized further to reveal their function and phenotype. Regardless of the purpose of determining a biomarker, especially in heterogeneous and evolving diseases such as cancer, cells *in vivo* do not always adhere to the rules that were followed when the marker was identified and evaluated *in vitro*. Therefore, biomarkers to identify, quantify and characterize heterogeneous tissues or bodily fluids should be used with caution to avoid under- or overestimation of the contribution of the cell type to the phenotype of the investigated cell population.

In this review, we will first elaborate on the biology behind tumor heterogeneity and how this affects clinical reality. Then, we will discuss the definition and applications of prognostic and predictive factors in cancer. We will discuss the various techniques that are currently available to isolate distinct cell populations from solid tissue and fluids, and finally the available methods for characterization of these cell populations down to the single-cell level.

1.1. The biology of tissue heterogeneity

Cancer is thought to be the consequence of clonal growth of one single cell containing one or multiple oncogenic somatic mutations. By the time a tumor lesion is detected, countless generations of daughter cells have been generated, and many pass on their acquired mutations to their individual clonal progeny. These acquired mutations occur at a rate much faster than would be expected when assuming mutation rates as observed in normal tissues. Many researchers assume that genomic instability, i.e., the adoption of a mutator phenotype which leads to an elevated rate of acquired mutations, is mandatory to generate the number of mutations that are necessary for malignant transformation. While the absolute necessity of genomic instability for the malignant transformation of a tumor has not been proven, evidence of genomic instability can be observed in cancer lesions [2] and has been linked to drug resistance in cell line models [3]. This drug resistance is thought to be augmented by the administration of

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cytotoxic treatment, as unstable cells are able to avoid cell cycle arrest after DNA damage while genomically stable cells do undergo cell cycle arrest and thus have a lower proliferation rate. Based on this reasoning, and only recently compellingly shown for solid tumors [4,5], a tumor consists of many sub clones with various and differing acquired mutations, leading to tumor heterogeneity.

This tumor heterogeneity has a profound impact on the optimal approach to biomarker analysis. When treatment decisions are being made based on the presence or absence of an amplified or mutated gene, but this gene is only mutated in a small clone with limited proliferative potential, one has to wonder whether we are currently truly employing "targeted therapy". Moreover, when basing treatment decisions on tumor biopsies or small fragments of a tumor, we cannot conclude that said phenotype is characteristic of the whole tumor. The lack of response that is observed even when selecting tumors based on activating mutations, such as *KIT* or *PDGFRA* in gastrointestinal stromal tumors (GIST), can be explained by the presence of a small clone with an additional downstream mutation of e.g. *KRAS* or *BRAF* [6]. This sub clone would then be able to circumvent the inhibitory effect of the targeted treatment, in this case imatinib, and has a survival advantage over the other sub clones. This example underlines the caution that needs to be employed when interpreting biomarker analyses of heterogeneous tissues.

1.2. The limitations of relying on a single marker

It is well accepted that gene expression differs in-between tissues. But also within tissues, even those of the same organ or tumor, there might still be a lot of heterogeneity [7]. An example of this is shown in Fig. 1 for the expression of EpCAM (epithelial cell adhesion molecule).

EpCAM is a cell surface molecule that functions as a homotypic calcium-independent cell adhesion molecule and is known to be expressed on most normal epithelial cells and epithelial tumor cells. The antigen is being used extensively as a marker for the enrichment of epithelial (cancer) cells from blood and, less extensively, for immunomodulatory treatment of human carcinomas [8]. Despite being a broadly expressed and well-validated marker, there is a risk in relying on only one target for the purpose of cancer detection and treatment. This has been demonstrated by work of our own group, in which we demonstrated that EpCAM-based isolation of circulating tumor cells does not enable detection of, in particular, normal-like breast cancer cells, which can have aggressive features [9].

Below we will now give examples – focused on the field of breast cancer with EpCAM as our example molecule, but applicable to different molecules and various kinds of cancer and diseases – of putatively suitable markers to evaluate tissue heterogeneity. We will also elaborate on how additional biomarkers can be identified by measuring the expression of mRNA gene transcripts in primary tumors of cancer patients. After this, it should become clear that there is still an urgent need for new predictive and prognostic biomarkers, especially those biomarkers that are specific for individual cell types present in a complex background mixture of cells.

1.3. Prognostic and predictive markers

To truly appreciate the putative wealth of information reflected by biomarkers, it is important to understand the difference between a prognostic and a predictive marker. In oncology, a prognostic marker predicts disease recurrence and ultimately survival in the adjuvant setting or tumor progression in the metastatic setting, independent of future treatment effects. A predictive biomarker predicts response or resistance to a specific cancer therapy. Any change in disease status during treatment should be reflected by a change in the marker status [10]. Prognostic factors, e.g. lymph node status, tumor size, histologic grade, proliferation index, ERBB2, hormone receptors and other biological markers are used to predict the clinical course of breast cancer at the time of primary treatment. Patients with a poor prognosis are offered radiotherapy and systemic adjuvant therapy in the form of

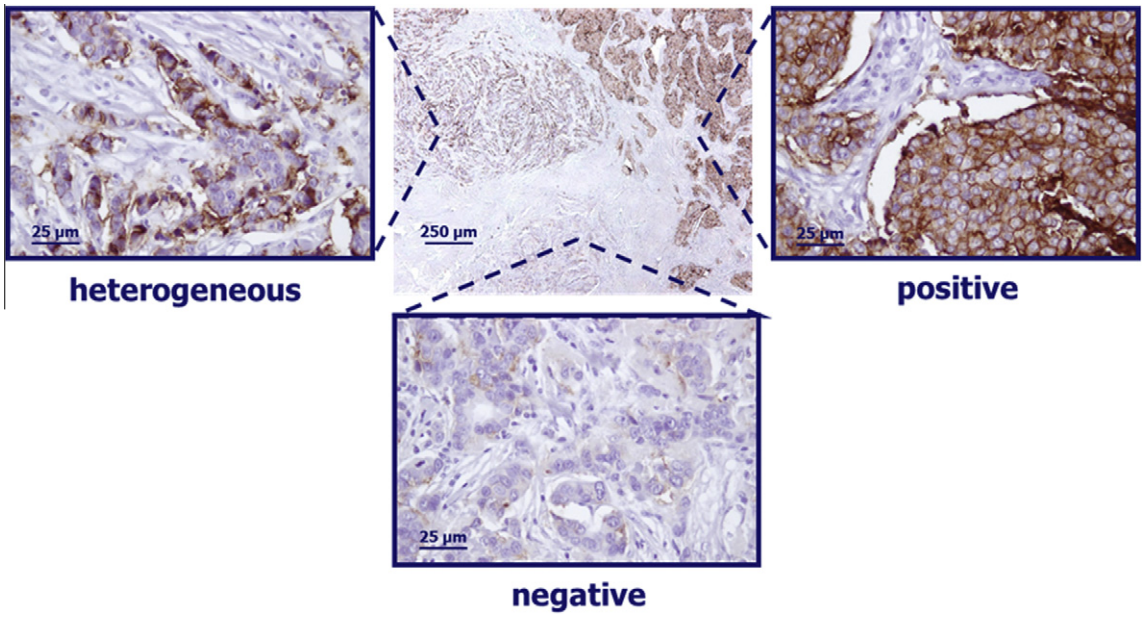

Fig. 1. Heterogeneous EpCAM staining in breast tumors. Example of heterogeneous epithelial cell adhesion molecule (EpCAM) staining within one invasive breast tumor with a normal-like phenotype [16]. Representative formalin-fixed paraffin-embedded tissue section of a normal-like human breast tumor stained for expression of EpCAM protein with a mouse-anti-human EpCAM antibody (clone VU1D9, Cell Signaling, Danvers, MA; 1:250 dilution, stained overnight after an antigen retrieval step in citrate buffer at pH 6.0; DAKO, Glostrup, Denmark). Anti-EpCAM (dark stain) was visualized with the peroxidase-conjugated Envision method from DAKO. The specificity of immunostaining was controlled by using normal mouse IgG and by omitting the primary antibody. Magnifications: middle overview=×40 and bar length = 250 µm; boxed detail sections=×400 and bar length = 25 µm [9].

endocrine, cytotoxic and/or biological anti-tumor therapy. Prediction refers to the likelihood of response to a specific therapy. For the choice of systemic adjuvant therapy, predictive factors such as age, ERBB2 amplification and hormone receptor status are useful, but are currently insufficient to truly select patients based on their response rate to specific therapies. For example, even in patients with a strongly hormone receptor positive primary tumor, a subset of patients is resistant to endocrine therapy, further underlining the need for more and better equipped predictive factors.

In general, biomarkers can be divided in circulating biomarkers that are detected in body fluids and markers detected in tumor tissue, with one new class of biomarkers, the circulating tumor cells, bridging the two. Examples of established breast cancer biomarkers are the serum-based cancer antigens CA 15-3, CA 125, carcinoembryonic antigen (CEA), the tissue-based estrogen and progesterone hormone receptors, circulating tumor cells (CTCs), several markers measuring DNA-ploidy/content and/or proliferation, the oncogene ERBB2, the tumor suppressor gene p53, and the protease urokinase plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor 1 (PAI-1) [11–14]. Before markers can be used in clinical decision making, they should be reported according to established guidelines as described in Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) [15], and be validated in well-designed prospective trials to provide Level Of Evidence 1 [10] justifying their use.

2. Discovery of cancer heterogeneity through gene expression profiles

The origin of heterogeneity is thought to lie within the evolutionary path each cell has to take before it reaches its final, differentiated destination. In this process, clonal selection and choosing advantageous conditions to survive are influenced by various factors such as the (micro) environment, hypoxia and other difficult conditions the cell has to deal with, and aging of the cell. All this will result in an accumulation of events affecting both the genotype and phenotype of the individual cells in a tissue or fluid, leading to heterogeneity. This tissue heterogeneity can be appreciated even with a simple morphologic evaluation, but its true extent has become apparent through the measurement of gene and protein expression profiling.

2.1. Molecular breast cancer markers

In order to estimate risk of cancer progression and to aid clinicians in choosing a patient-tailored therapy, molecular profiling has been used extensively in recent years. Because these can reflect the heterogeneity among tumors, microarray-based molecular classification of tumors is now considered to be at least complimentary to established clinical and pathological criteria such as the aforementioned guidelines and prognostic tools.

The first well-known studies in this field, aimed at classifying breast carcinomas based on gene expression patterns, are those of Perou and Sorlie [16–20]. According to these studies, breast carcinomas can now, based on patterns of expression of 496 ''intrinsic'' genes, be distinguished in tumor subclasses with distinct clinical implications:

(I) Luminal-epithelial group (with sub-classification into types A–C), characterized by expression of the estrogen receptor and genes associated with the estrogenic function, i.e., genes that are typically expressed in the luminal epithelium that lines the ducts.
(II) Basal-epithelial group, typically negative for the estrogen and progesterone receptors as well as the ERBB2 (Her2/neu) oncogene (''triple negative'').
(III) ERBB2 + group associated with over expression of the ERBB2 amplicon.
(IV) Normal breast-like group, a group of tumors bearing a close resemblance to normal breast cancer cells but with aggressive features.

Additionally, the Genomic Grade Index (GGI), capturing 97 differentially expressed genes, can be used to distinguish patients with a high versus a low risk of recurrence among patients with histologic grade 2 tumors [21,22]. These two examples represent a new class of biomarkers using gene expression profiles to classify breast cancer. Additional examples of the numerous publications based on the use of molecular profiles that appeared in the early 2000's were those aimed at identifying:

- histological subtypes [23–32],
- subgroups with different prognosis [25,33–50],
- subgroups with different sites of relapse [51–54],
- subgroups with different response to treatment [49,55–75] and
- circulating tumor cells [76–82].

Despite the overwhelming number of publications, only a few of these profiles – such as MammaPrint and Oncotype DX for breast cancer patients [83] – are now implemented into clinical practice. Other molecular profiles still require extensive prospective validation. Ultimately, the presence of drug targets and targetable signaling pathways rather than molecularly defined subgroups will most likely drive treatment decisions [50,84]. However, in the interpretation of all these profiles, we need to bear in mind that their results are based on nucleic acids isolated from complex tissues, and are therefore only informative of the dominant cell types, while a subpopulation of less abundant cells might be more indicative of prognosis or tumor response.

3. The choice of sample for marker evaluation

Clinical samples can be divided into two major types: solid tissues and liquid samples. Solid tissues usually concern either biopsies or surgical resection specimens. They can be stored in liquid nitrogen to stay fresh-frozen (FF) or alternatively, they can be embedded in paraffin after being fixed overnight with formalin (FFPE). The method of preservation will affect the choice of suitable techniques available to study the molecules present in these complex and heterogeneous materials.

When looking at solid cancer tissues, another important distinction with regard to the most optimal sample can be made between primary tumor and metastases. As discussed before, tissue heterogeneity is a natural consequence of processes within a tumor such as clonal selection and genomic instability. These processes, and importantly also the process of epithelial-to-mesenchymal transition (EMT) which tumor cells are thought to undergo upon entering the blood stream, lead to discrepancies between the primary tumor tissue and corresponding metastases. Especially when much time has passed and systemic anti-tumor therapy has been administered, the latter of which might lead to selection of resistant cell populations, these discrepancies can have direct clinical consequences. Recently, many studies have focused on this heterogeneity between primary tumor and metastases, and single-cell sequencing has revealed the complexity of tumor evolution [4,5]. These differences between primary tumors, which are now most often used for biomarker analysis, and metastases, which pose the biggest threat to the patient, probably means that determining prognostic and predictive factors on metastatic lesions leads to

better treatment decisions. The marked heterogeneity even within a metastatic lesion, however, also raises the question whether metastatic biopsies truly reflect the patient's tumor load. In this sense, circulating tumor cells, which are thought to be aggressive tumor cells representing metastatic lesions, are a promising alternative (see Section 4.3).

Besides solid tissues, which are notoriously composed of multiple cell types, several types of liquid samples are available for research and clinical diagnostics, such as blood, bone marrow, urine, saliva, central spinal fluid (CSF), pleural effusion samples (PES), peritoneal effusion samples (ascites) and amniotic fluid samples (AFS). Despite their usually less complex nature, fluidic samples too contain multiple cell types, influencing the types of analysis that can be done on these samples.

Below, we will discuss the different available methods to isolate cell populations from heterogeneous samples, divided into those available for solid and liquid samples.

4. Isolating individual cell types from heterogeneous material

Separation of cells requires the removal of one cell type from another by physical means. Depending on the type of the sample, there are several methods available to separate cells.

4.1. Solid tissues

4.1.1. Physical tissue separation

In order to select for specific cell populations in heterogeneous tissues, several microdissection techniques have been described. Most techniques involve the use of a needle to scrape off cells of interest under direct microscopic visualization [85,86]. This method, however, tends to be slow, tedious and highly operator dependent [87]. In 1992, Shibata and coworkers described a new method for cell isolation. They used a specific pigment placed over small numbers of cells in a tissue section, which served as an umbrella preventing the covered cells from being destroyed by the ultraviolet light which was used to destroy the DNA/RNA of the uncovered cells [88]. Shortly later, laser capture microdissection under direct microscopic visualization was developed by Liotta and coworkers in the National Cancer Institute (NCI). This way of target cell isolation permits rapid, reliable laser microdissection to collect specific cell populations from a section of complex, heterogeneous tissue [89]. There are several membranes and caps used with laser capture microdissection (LCM) [90]. Dependent on the microlaser dissection device used, the collection caps used are positioned in different ways. LCM is compatible with a variety of cellular staining methods and tissue preservation protocols [91], making LCM a powerful method to procure subpopulations of tissue cells under direct microscopic visualization. This technology can harvest the cells of interest directly or can isolate specific cells by cutting away unwanted cells to give histologically pure enriched cell populations [92]. The LCM process does not alter or damage the morphology and chemistry of the sample collected, nor of the surrounding cells. For this reason, it is a useful method to collect selected cells for DNA, RNA and/or protein analyses [93]. In addition, it is specific enough to dissect single cells and – with proper measures taken – can yield good quality samples. The collection of large quantities of cells by LCM is a time consuming procedure requiring the microscopical visualization of the cells of interest in a stained tissue sections before lasering [90].

One of the recently most used LCM devices is the PALM micro laser dissector (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) (Fig. 2).

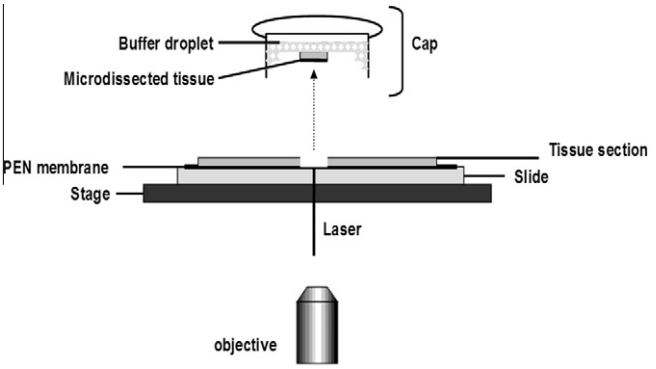

Fig. 2. P.A.L.M. laser capture micro-dissection technique. The PALM provides a powerful separation technique, in which the laser microbeam microdissection (LMM) combined with laser pressure catapulting (LPC) is an important improvement over the more traditional UV-laser based cutting techniques [146]. Furthermore, specific glass slides covered with a polyethylene naphthalate (PEN) membrane aid in stabilizing the morphological integrity of the captured area [147]. In this method, collecting caps do not make contact with the tissue sections, which increases the flexibility with respect to section preparation [148].

4.1.2. Enzymatic & mechanical tissue disruption

This method includes disturbing the extracellular matrix and cell adhesion components without harming the integrity of cell membrane. This technique needs a combination of various enzyme mixes, mechanical forces, incubation periods and temperatures [94]. Nowadays, there are several robust instruments available on the market to enable the effective disruption and homogenization of samples. This step is usually followed by enrichment with magnetic beads covered with an antibody that recognizes the cells of interest [95]. Alternatively, the mechanical disruption and separation of cells may be followed by flow cytometric classification of cells based on antigen expression [96]. This approach enables the isolation of high quality RNA, DNA and proteins.

4.2. Liquid samples

Liquid samples like urine, blood or bone marrow don't need mechanical disruption. Depending on the sample type, an enrichment step usually precedes the detection method of choice. We will discuss various options for enrichment of specific cell types and their subsequent detection in liquid samples, using circulating tumor cells (CTCs) as a final example.

4.2.1. Differential centrifugation

Differential centrifugation is a very common enrichment procedure of separation based on size and density, with larger and denser particles pelleting at lower centrifugal forces. This technique enables the enrichment for some cellular components like nuclei, mitochondria, ribosomes and others, thus, extracting the specific genetic material from each component separately. It is however associated with significant cell loss, which, in the analysis of rare cell populations, can be a serious disadvantage.

4.2.2. Density gradient separation

This is one of the best established cell separation techniques. It is performed by cell density gradient centrifugation on a ficoll–hypaque solution. This method was developed in 1968 by Bøyum, using low viscosity Ficoll and sodium metrizoate of the proper density and osmotic strength, to isolate mononuclear cells [97]. Later, sodium metrizoate has been successfully substituted with sodium diatrizoate [98]. The basis for this cell separation assay is the differential migration of cells during centrifugation according to their buoyant density, which results in the separation of different cell types into distinct layers [99] (Fig. 3).

One of the advantages of this method, besides its speed and low cost, is the separation of erythrocytes by the porous barrier plug,

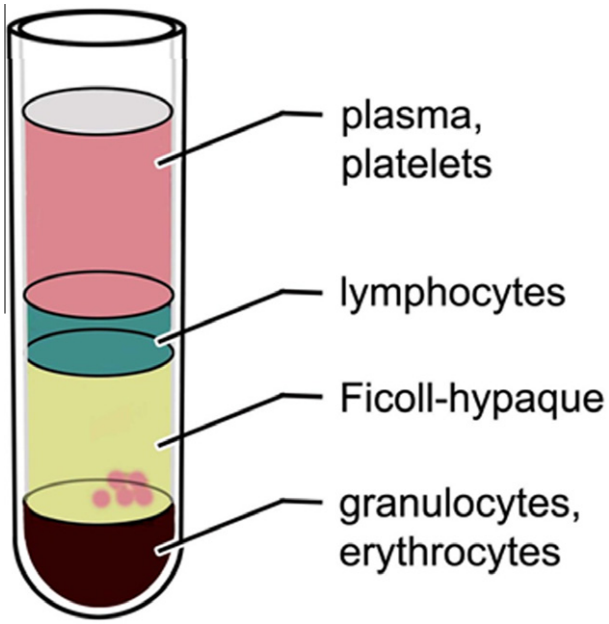

Fig. 3. Density gradient separation. An example of the different cell layers obtained after density gradient separation of a whole blood sample. The red cell pellet, including the granulocytes, is separated from the lymphocytes by the ficoll–hypaque layer. The top layer consists of plasma and platelets, which can be distinguished based on its clarity as compared to the lymphocyte layer [149].

which makes it easy to separate the nucleated cells of interest. However, like differential centrifugation, it is associated with some loss of cells.

4.2.3. Filtration

This physical separation method is based on using filters with specific pores that separates cells of interest from other cells based on their size. Tumor cells are presumed to generally be bigger in size than other peripheral blood mononuclear cells (PBMCs), and are thus trapped on top of the filter while the other cells are allowed to pass through [100]. While the big advantage of these size-based methods is the circumvention of the need for a marker that is expressed on all cells of interest, also cell size can be heterogeneous among the cell population of interest. One method based on filtration as an enrichment step for CTCs is further explained in Section 4.3 and Fig. 4.

4.2.4. Antibody-based separation

Antibody-based separation relies on the expression of specific antigens on the cell surface or within the cytoplasm of cells of interest. This is a good and powerful method because of the great diversity and specificity of antibodies, but its success depends completely upon the choice of antibodies. The expression of a cell-specific antigen can be used to separate antibody-bound cells from other cells by immunomagnetic enrichment or depletion. By addressing multiple, putatively differentially expressed targets, this can result in a simultaneous enrichment based on different antigens such as in the AdnaTest (AdnaGen AG, Laggenhagen, Germany), thereby increasing the sensitivity of this enrichment step. The actual immunomagnetic enrichment step can be manual in a hand-held magnet, or semi-automated such as in the CellSearch technique (Veridex LLC, Raritan, NJ) (see below).

The cell-specific expression of certain antigens can also be employed in flow cytometry assisted cell sorting (FACS), which allows for simultaneous separation of various specific cell populations through the combination of several fluorescently-labeled antibodies.

4.3. Circulating tumor cells

As an example for the intricate combination of highly sensitive enrichment and extremely specific detection techniques to isolate rare cells from a heterogeneous cell population, we will now focus on methods available for the isolation of circulation tumor cells (CTCs).

CTCs are cancer cells that have detached from a solid tumor and entered into the bloodstream. They have for a long time been considered a promising biomarker enabling repeated and non-invasive assessment of prognostic and possibly predictive factors. As a result, many attempts have been made to develop assays that can reliably detect and enumerate CTCs, but only recently such assays have become available on the market. The first clinical results obtained with such assays strongly suggest that in a variety of tumor types, including breast, colorectal, prostate and lung, CTC enumeration can be used to predict prognosis, and a second CTC count can serve as an early marker to assess anti-tumor activity of a given treatment [101]. The detection of CTCs remains extremely challenging due to their low number in the blood as compared to PBMCs; median CTC count in metastatic disease ranges from 3 to 5 cells per 7.5 mL blood, underlining the need for technologically advanced assays.

So far, the CellSearch™ is the only assay that is approved by the US Food and Drug Administration (FDA) for use as a prognostic marker in metastatic breast, colorectal, lung and prostate cancer. In this semi-automated method, CTCs are isolated from whole blood by immunomagnetic enrichment using ferrofluids coated with antibodies against epithelial cell adhesion molecule (EpCAM). Subsequently, the isolated cells are stained with fluorescently-labeled monoclonal antibodies targeted against cytokeratins (CK) 8, 18 and 19, selecting for epithelial cells, CD45 to exclude leucocytes and a nuclear staining dye (DAPI), and enumerated using a semi-automated fluorescence microscope. In this method, CTCs are defined as nuclear cells expressing CK 8, 19 or 19 and lacking CD45 expression [102]. The prognostic value of CTCs as enumerated by CellSearch has clearly been shown, but due to the nature of the assay, i.e., selection based on epithelial-specific markers, improvements in the sensitivity of this assay are most likely possible. In our own work, we have demonstrated that breast cancer cells of the normal-like subtype lack EpCAM expression and are thus missed using the conventional CellSearch technique [9], a problem that can for the most part be overcome by adding CD146 as an enrichment marker [103]. For the purposes of CTC characterization, CTCs can also be isolated from whole blood using only the EpCAM-based immunomagnetic enrichment step from CellSearch without further staining. While this enrichment enables the large-scale gene expression analysis of CTCs [78,104], up to a thousand background leukocytes are still present [104]. This requires a careful selection of CTC-specific genes that are preferentially not at all or else much lower expressed by leukocytes [78,104].

We will now discuss some of the more prominent new alternative CTC detection techniques. A complete overview would be beyond the scope of this review, and we refer to Mostert et al. for a more elaborate discussion of currently available detection techniques [105].

EPISPOT is an immunological assay derived from the enzyme-linked immunosorbent assay (ELISPOT) [106]. Before detection of CTCs, leukocyte content is minimized through immunomagnetic anti-CD45-based depletion and tumor cells are selected based on CXCR4-expression. CXCR4 is a chemokine receptor involved in the homing of metastatic tumor cells, and is thought to be expressed on CTCs. EPISPOT then detects specific proteins released

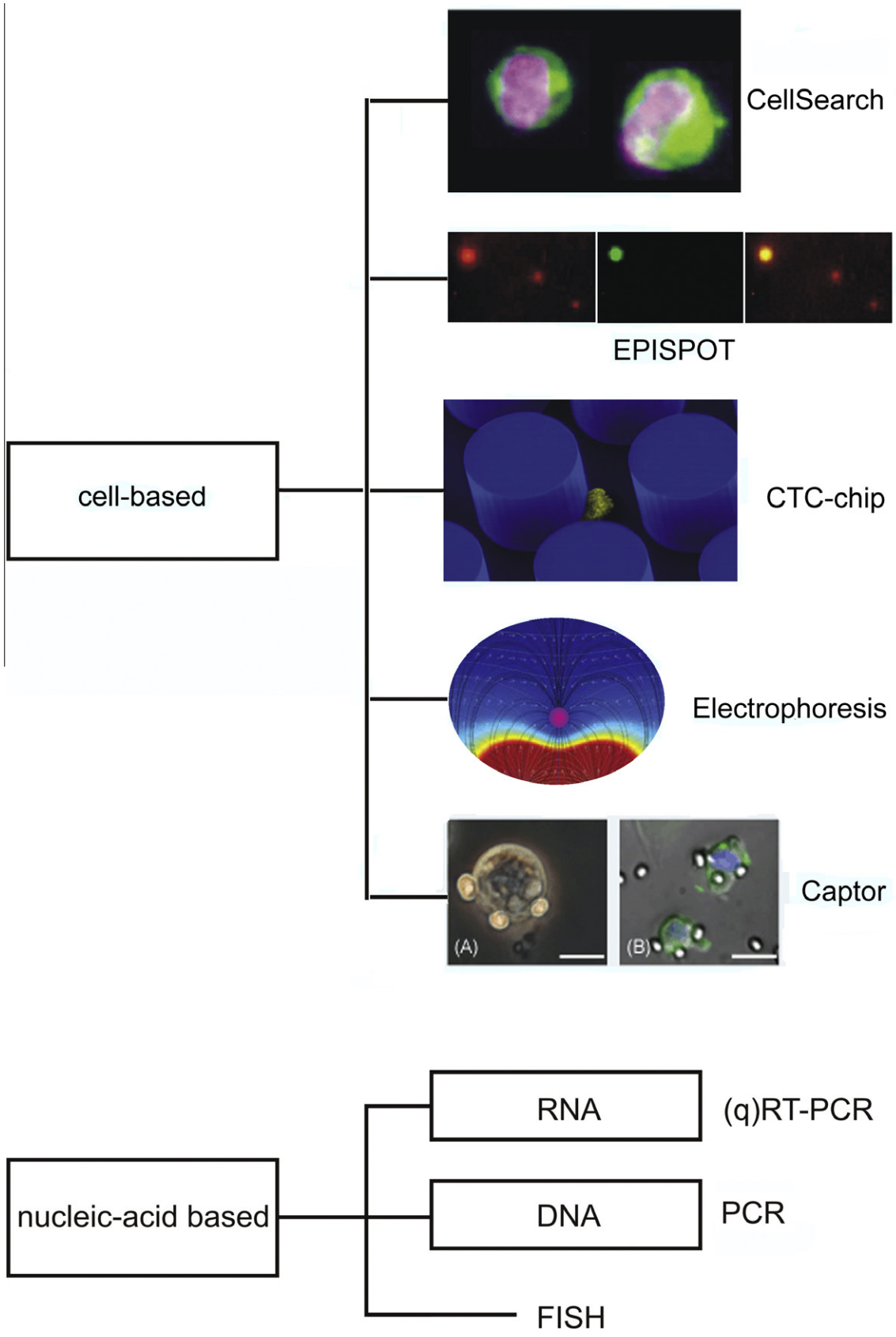

Fig. 4. Overview of cell-based (top panel) and nucleic-acid based (bottom panel) methods currently in use for the detection of CTCs. Adapted from Mostert et al. [105]; lower part of the figure published with permission of Abnova [<http://www.abnova.com/system/captor.asp>].

by viable CTCs, such as Cathepsin-D or Mucin-1, leading to an enumeration of only viable cells. Unfortunately, this method does not enable morphological confirmation of CTCs and, like CellSearch, depends on the extent to which the markers used are expressed or released by all CTCs.

The CTC-chip is one of a few available methods based on the attachment of monoclonal antibodies to microposts on a chip [107]. Results have been described for the CTC-chip with microposts coated with EpCAM [108] and with PSA antibodies [109]. The advantage of this method is the controlled flow by which whole blood is pumped across the chip, increasing the possibility of a CTC to bind to one of the 78,000 microposts on the chip. After binding of CTCs to the microposts, they are stained against CKs and DAPI for positive selection and CD45 for negative selection. Remarkably high CTC counts were described with this method as compared to other EpCAM-based methods, which have thus far unfortunately not been reproduced. Because EpCAM has been described not to be expressed on all breast cancer subtypes [9], caution is needed when interpreting these results.

In an effort to circumvent the need for a marker expressed on all tumor cells, numerous assays have been described using dielectrophoresis to select CTCs [110,111]. These methods exploit the intrinsic properties of tumor cells; most notably the cell membrane capacitance, a measure of plasma membrane area, and conductance. These unique properties cause tumor cells to transit the assay chamber at different speeds, and separate them from other blood cells. While assay sensitivity might not be optimal with these methods and current assays are limited to low blood volumes, their crucial advantage is the absence of any specific enrichment or detection markers and their ability to obtain viable tumor cells. However, morphology too can be heterogeneous among cells of interest. Indeed, circulating tumor cells have been described to vary in size, some not exceeding the size of PBMCs, and these PBMC-sized CTCs were also prognostic in prostate cancer patients [112].

A new device to detect CTCs is the Captor™ device, which filters tumor cells from other PBMCs based on their bigger size and lesser deformability, and then selects these cells based on EpCAM and DAPI expression in the absence of CD45 expression. This method does not require permeabilization of cells, which is needed for cytokeratin staining, and thus viable CTCs are retrieved. High isolation purity was obtained with this method in cell line spiking experiments [113], but the added value of this method in patient samples remains to be elucidated.

The presence of CTCs, or nucleic acids derived from CTCs, can also be detected and quantified in blood with molecular techniques such as PCR, real-time quantitative reverse transcriptase PCR (qRT-PCR) [113] or fluorescence *in situ* hybridization (FISH) [114], methods based on the detection of (epi)genetic alterations that are specific for cancer cells. The differential expression of specific mRNAs [113] or presence of oncogenic mutations [115] can thus be identified in whole blood or plasma. In these methods, high specificity is even more crucial than in cell-based methods, as morphological confirmation of CTCs is not possible. This specificity can be obtained by first selecting only tumor cells based on a powerful enrichment step and/or by carefully selecting genes of interest based on the absence of their expression on PBMCs. By enriching for EpCAM-positive cells and measuring only epithelial-specific genes, we have thus for example succeeded in measuring 55 breast cancer CTC-specific mRNAs and 10 breast cancer CTC-specific miRNAs in as little as one cell per 7.5 mL blood [113].

5. Biomarker detection in heterogeneous samples

Biomarkers can be found in a variety of fluids and tissues. Genomic and proteomic technologies have greatly increased the number of potential DNA, RNA and protein-related biomarkers under investigation. A paradigm shift has recently been realized, whereby single-biomarker analysis is being replaced by multiparametric analysis of genes or proteins as a molecular signature. In relation to tumor heterogeneity, these molecules can be produced either by the epithelial (tumor) cells themselves, or by cells present in the surrounding tissue like the stroma. In the case of stroma, molecules comprising tumor-specific signatures can be expressed in response to the presence of cancer or due to other biological conditions, such as inflammation. An ideal tumor marker should be easily, reliably and cost-effectively measurable by use of an assay with high analytical sensitivity and specificity [116]. However, despite numerous efforts, only a few markers in a limited number of cancer types have been able to meet those stringent conditions and have successfully entered routine daily practice.

Currently, most biomarkers with clinical applications include either genetic or proteomic markers [117]. The development of new technologies, such as gene expression profiling, proteomic approaches and next generation sequencing technologies, in combination with *in situ* hybridization and immunohistochemistry, have enabled researchers to screen the whole genome, proteome, transcriptome and metabolome for new biomarker discovery and validation in (tumor) tissue, serum, plasma, or other human body fluids [116]. We will now discuss the specifications, limitations and applications of some of these biomarker discovery techniques, with a focus on the analysis of small samples down to the one cell level.

5.1. Genomics

5.1.1. (Quantitative) RT-PCR

With a long history at the single biomarker level, (q)RT-PCR is generally considered the 'gold standard' against which other methods are validated. QRT-PCR is a suitable technique even for a single cell that contains ~25 pg total RNA, comprising ~0.5 pg mRNA, which is equivalent to ~500,000 mRNA's of average size. This technique is relatively easy, reliable and quantitative. It is splited into two major steps. The first step requires a highly sensitive reverse transcription followed by an accurate step of amplification with a wide dynamic range (1000-fold differences can be measured), and can be highly reproducible [119]. In addition, with (q)RT-PCR one can simultaneously measure the expression of multiple genes in a multitude of single-cell samples. However, qRT-PCR data constitute only a snapshot of information regarding the quantity of a given transcript in a cell or tissue. Any assessment of the biological consequences of variable mRNA levels must include additional information regarding regulatory RNAs, protein levels and protein activity [119]. For this, new technologies are now fortunately available in this era of high throughput profiling by micro-array and deep sequencing technologies.

5.1.2. Micro-arrays

During the past decade, the most successful and widely used whole transcriptome analysis method for large-scale biomarker discovery was the cDNA microarray [120–122]. Genomic microarrays represent a highly powerful technology for gene-expression studies because of their ability to analyze a multitude of genes simultaneously in one sample. Microarray experiments are usually performed with DNA or RNA isolated from tissues, which are labeled with a detectable marker and allowed to hybridize to arrays comprised of gene-specific probes that represent thousands of individual genes in order to assess the relative gene expression level [123]. To meet the demand of the research community to be able to evaluate individual cells in these heterogeneous tissues, single-cell methods have been developed for DNA microarrays

[124,125]. The huge amount of data per experiment requires appropriate statistical analysis tools to discover which genes, up or down regulated, might be considered as potential biomarkers. In cancer research, unsupervised and supervised hierarchical clustering algorithms are the most frequently used tools to generate gene signatures, which are then capable of classifying tumors based on predefined clinical information [126].

One of most successful examples of gene-array technology is the classification of breast cancer into prognostic categories dependent on the expression of certain genes [37,42]. Studies showing the ability of a 70-gene-panel measured by microarray to predict survival in primary breast cancer patients led to the development of MammaPrint® (Agendia, Amsterdam, The Netherlands) [37]. This panel became the first multigene panel test to be approved by the FDA for predicting breast cancer relapse in February 2007. Another multigene signature, Oncotype DX® (Genomic Health, Redwood City, CA), based on qRT-PCR of 16 cancer-related genes and 5 reference genes has been commercially available since 2004 for a similar use; predicting the recurrence of tamoxifen-treated, node-negative breast cancer [58]. The true value of these signatures will be elucidated in two studies assessing the prognostic value of Oncotype DX and MammaPrint prospectively, and these results are awaited before a definitive implementation into the clinic can be initiated. Nonetheless, over the past decade, there has been a tremendous growth in the application of the gene-expression array technology, resulting in the classification of breast cancer according to intrinsic subtypes [16], insights into cancer pathogenesis, and the discovery of a large number of diagnostic markers [127].

5.1.3. RNA sequencing

Despite these promising studies, DNA microarray technology has significant limitations [128], including (1) cross-hybridization between genes of similar sequence; (2) not all genes are reliably detectable, especially those with a low expression level; (3) lack of information about the exact length and sequence of the mRNAs being analyzed; and (4) the inability to detect novel transcripts. In most gene-expression studies, microarrays are now being replaced by RNA sequencing (RNA-Seq) based methods which directly determine the complete cDNA sequence and can be used to identify and quantify mRNA transcripts, non-coding RNAs, small RNAs, splicing patterns and other post-transcriptional modifications. This technology can also provide information about mutations, insertions, deletions, SNPs, copy number variation and chromosome rearrangements [128,129], thereby greatly increasing the information obtained from a tumor sample as compared to microarray technologies.

The RNA-Seq procedure is simple and requires only small amounts of nucleic acid material (~100 ng of total RNA), but generates an immense quantity of data. It has a large dynamic range and high sensitivity, and can unequivocally identify splicing and RNA editing products as well as allele-specific transcripts [128]. Additionally, with the current second generation sequencing technologies, we can easily determine the polarity of the transcripts, which is important given the fact that many genomic regions give rise to transcripts from both strands [130–133]. This problem has now been solved with the use of smart protocols for strand-specific RNA sequencing [134], which can be used in combination with the different platforms available in the market like SOLID, Illumina, and Roche 454. In the future, these RNA-seq protocols need to be updated to be used by the third generation sequencers which aim to increase throughput and decrease the time to result and cost, like the Ion Torrent or Pacific Bioscience platforms. At the analysis side, progress can be made by simplifying the statistical analysis of these huge amounts of data through the generation of new algorithms and software packages. Altogether, because of its high-quantity and high-quality data output, RNA-Seq will definitely contribute to the discovery of new biomarkers. Additionally, previously described biomarkers can be reliably validated on large scale data sets for validation.

Fig. 5 shows a representative example of EpCAM strand specific RNA-sequencing of both fresh frozen (FF) and FFPE SKBR-3 breast cancer cell line cells. Gene expression levels are expressed in reads per kilobase of exon model per million mapped reads (RPKM). The RPKM measure of read density reflects the molar concentration of a transcript in the starting sample by normalizing for RNA length and for the total number of reads in the measurement [133]. RPKM values for EpCAM were very comparable in both samples, underlining the ability to use both fresh frozen and FFPE samples in this assay. To validate the RNA-seq expression data, we compared these results with data we obtained with the WG-DASL (Whole-Genome cDNA-mediated Annealing, Selection, Extension and Ligation) assay, an assay specifically equipped to measure transcripts in highly degraded material such as FFPE. Using the data obtained with the EpCAM Illumina probe ILMN_2160209 on RNA isolated from both FF and FFPE material of the SKBR-3 cells, results for these two sample types and methods to evaluate transcript levels, were very comparable.

5.1.4. Single-cell analysis

In complex tissues, heterogeneity may indicate the presence of specialized cell types or originate in the random nature of the transcription machinery [135,136]. The above described technologies in the "omics" era produce a large number of possible biomarker candidates, which require proper validation before clinical application. It is in fact the large heterogeneity between and within individual tumors, together with highly variable expression levels across the human population, which makes the validation of these biomarkers extremely challenging. To overcome this issue, researchers can now decide to isolate the separate cell populations, down to the one cell level, which increases assay specificity but does demand a higher sensitivity of the techniques. Furthermore, when purifying DNA and RNA from very few cells, the issue of stochastic variation becomes important. For example, if 10 cells are processed, and DNA is eluted in 25 μL, there will be less than 1 copy of each genomic DNA allele per micro liter. Similarly, some RNA transcripts may be present at very low copy numbers per cell, or only in a fraction of all cells in the sample of interest.

As mentioned before, a typical single cell contains ~0.5 pg mRNA, which is equivalent to a few hundred thousand molecules transcribed from about 10,000 genes [118]. qRT-PCR is a suitable technique for single-cell gene expression because it has a wide dynamic range (1000-fold differences can be measured), is highly reproducible and can simultaneously measure the expression of multiple genes in a multitude of single-cell samples. Multiplexed mRNA expression analysis with single-cell resolution is also possible using FISH, which has the additional benefit of enabling morphological analysis, but the degree of multiplexing is generally limited to 3–5 targets [137]. As an alternative approach, single-cell methods have been developed for DNA microarrays [124,125] followed by single-cell RNA-seq technologies to successfully analyze a single cell transcriptome [138–140]. Recently for example, Xu et al., published results of a single-cell exome sequencing experiment from a renal cell carcinoma patient, aiming to better understand the intratumoral genetic underlying mutations of this tumor [141]. They carried out exome sequencing of 25 single cells from the tumor and adjacent noncancer kidney tissue. They were able to cover 100% of the exons of the *VHL* and *PBRM1* genes, which are the most commonly mutated genes in renal cell carcinoma. The new technique facilitated to conclude the cancer in that patient was unlikely to be related to the presence of *VHL* and *PBRM1* mutations and would most likely not benefit from a therapy

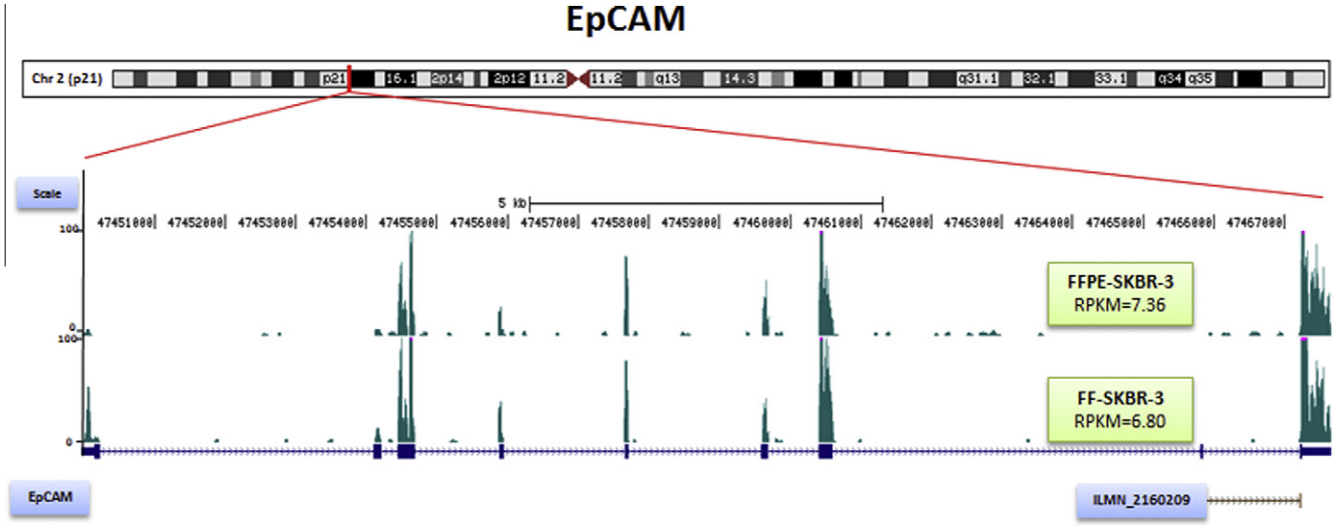

Fig. 5. UCSC genome browser EpCAM read counts form formalin fixed and paraffin embedded (FFPE) and fresh frozen (FF) SKBR-3 breast cancer cell line. After rRNA depletion and RNA fragmentation of 100 ng of total RNA, cDNA was generated by random priming following the strand specific protocol. Then, the cDNA was converted into a molecular library for the Illumina GAxII by a single end sequencing protocol, after which the resulting 35-bp reads were mapped onto the reference genome hg18. The gene expression data given by the RPKM values were comparable between both samples, and compared well with the gene expression data as measured by the EpCAM ILMN_2160209 probe included on the WG-DASL chip.

targeted against these mutations. Therefore, they concluded, investigating other genetic mechanisms that may underlie this type of renal cell carcinoma may lead to new ways to investigate individual tumors, with the aim of developing more effective cellular targeted therapies.

5.2. Biomarker validation and heterogeneity

In complex tissues, heterogeneity may indicate the presence of specialized cell types or originate in the random nature of the transcription machinery [135,136]. The above described technologies in the "omics" era produce a large number of possible biomarker candidates, which require proper validation before clinical application. One method to validate the precise localization of putative biomarkers within an heterogeneous tissue is to make use of tissue microarrays (TMA). TMAs are arrays of core biopsies obtained from FFPE tissues and can thus provide high-throughput gene (by *in situ* hybridization) or protein expression analysis of large cohorts of cancer patients on a single slide. Some biomarkers have already been validated in colon cancer and breast cancer using this promising technology [142,143]. While the major advantage of TMA is that analysis of tumors from many different patients with different stages of disease can be performed simultaneously, the major drawback of this technique is that, because of the small punch size, TMAs may not demonstrate tumor heterogeneity, which can commonly be estimated in whole section mounts. Therefore, the choice of the tumor area is pivotal and, in the case of widely heterogeneous tumor, like prostate and breast cancer, numerous punches may be necessary [144].

It is in fact the large heterogeneity between and within individual tumors, together with highly variable expression levels across the human population, which makes the validation of these biomarkers extremely challenging. In breast cancer, HER-2 overexpression for example is a predictive marker of tumor aggressiveness and therapy response. In advanced and metastatic breast patients HER-2 overexpression identifies the subset of patients who can benefit from trastuzumab. In general HER-2 is measured in the primary tumor, even if the metastases appear several years later. There are several studies that demonstrate widely discordant rates in HER-2 overexpression between the primary tumor and metastases of the same breast cancer, emphasizing the existence of biological differences between primary tumors and their metastases. Thus, evaluation of HER-2 in metastatic sites, especially when the primary tumor was negative, can improve trastuzumab benefits in HER-2 positive metastatic breast cancer patients [145].

To overcome these issues, researchers can now decide to isolate the separate cell populations, down to the one cell level. This increases assay specificity, but does demand a higher sensitivity of the techniques. Furthermore, when purifying DNA and RNA from very few cells, the issue of stochastic variation becomes important. For example, if 10 cells are processed, and DNA is eluted in 25 μL, there will be less than 1 copy of each genomic DNA allele per micro liter. Similarly, some RNA transcripts may be present at very low copy numbers per cell, or only in a fraction of all cells in the sample of interest.

6. Key issues, pitfalls and challenges

Heterogeneity touches the very essence of cancer as a disease; it is caused by its inherent genomic instability and the presence of specific cell types, and is an important factor in the lack of uniform response to anti-tumor treatment seen in cancer patients. It is this heterogeneity that sparks the need for reliable prognostic biomarkers to distinguish patients needing aggressive therapy from those benefitting more from a wait-and-see approach and predictive biomarkers to select patients most responsive to a certain targeted treatment. Conversely, the same heterogeneity complicates the identification of such prognostic and predictive biomarkers from tumor tissue, fluid samples or circulating tumor cells.

Especially in heterogeneous and progressive diseases such as cancer, cells *in vivo* do not always adhere to the rules that were followed when the specificity of the marker was evaluated. Therefore, the use of only one biomarker to identify and quantify specific cells in a tissue or fluid is likely to be prone to under- or overestimation of the contribution of the cell type.

When a search for a new biomarker is initiated, it is crucial to predefine the kind of information it should give, in what patient population and at which time point in the course of the disease it can be used and what criteria should be met in terms of assay sensitivity and specificity. For instance, it is impossible to look for a reliable prognostic factor in a heterogeneously treated patient

population, just like it is extremely challenging to look at rare stem-cell like features in cell populations without a sensitive enrichment step.

Much progress has been made in the ability to select cell populations of interest from a larger heterogeneous cell population, both in solid tissues by laser microdissection and by size-, density-, or antibody-based enrichment in fluid samples and CTCs. These enrichment steps can be followed by sensitive high-throughput genomics and proteomics detection methods such as cDNA microarray, qRT-PCR, RNA-sequencing, mass spectrometry and PLA. While all very sophisticated, the success of these assays relies completely upon the correct choice of biomarker, i.e., a biomarker that is sensitive and specific enough for the cell population of interest.

It is only by further classifying patient populations as well as individual tumor samples into distinct subtypes that we can move forward towards our aim of patient- and tumor-tailored treatment, and it is of the utmost importance that researchers take into account the effect of tumor and patient heterogeneity in their research questions.

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