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Roberto Piñeiro Editor

Circulating Tumor Cells in Breast Cancer Metastatic Disease



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Circulating Tumor Cells in Breast Cancer Metastatic Disease

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Editor Roberto Piñeiro Roche-Chus Joint Unit, Translational Medical Oncology Group (Oncomet) Health Research Institute of Santiago de Compostela Santiago de Compostela, Spain

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Introduction – Biology of Breast Cancer Metastasis and Importance of the Analysis of CTCs

Roberto Piñeiro

Abstract

Breast cancer metastasis is a complex multistep process during which tumor cells undergo structural and functional changes that allow them to move away from the primary tumor and disseminate to distant organs and tissues. Despite the inefficiency of this process, some populations of circulating tumor cells (CTCs), which are those cells responsible of metastases formation, are able to survive in blood circulation and grow into secondary tumors. Metastatic breast cancer remains an incurable disease, and the phenomenon of metastasis represents the larger cause of death in these patients. The application of liquid biopsy techniques and the advancements in the field have shown the prognostic value of CTCs, suggesting the importance that CTCs analyses may have in the clinic. However, their implementation in routine clinic has not been yet achieved due to the yet small body of evidence showing their clinical utility. This introductory chapter will revise the key aspects of breast cancer metastasis and discuss the importance of CTC analyses in the management of breast cancer patients.

Keywords

Breast cancer · Metastasis · Circulating tumor cells (CTCs) · Tumor heterogeneity · Liquid biopsy

1.1 Breast Cancer Metastasis

Breast cancer (BC) is the most prevalent cancer among women worldwide. Surgical resection of the primary tumor has an elevated successful rate in early-stage BC, however 20–30% of patients will eventually develop disseminated disease or metastasis [1], resulting in the leading cause of cancer deaths. Despite advances in screening, diagnosis and treatment, a significant proportion of patients is diagnosed in advanced stages, with a median survival of ranging from 2 to 3 years for stage IV disease [2], depending on type and site of disease.

Breast cancer is divided into different subtypes according to the molecular profile of the tumor; the estrogen receptor positive (ER+)/progesterone receptor positive (PR+) subtype, also known as luminal; the HER2+ (human epidermal growth factor receptor positive) subtype, and the triple-negative (TNBC) subtype, which lacks the expression of ER, PR and HER2. The different molecular subtypes have implications on the systemic treatment planning. Thus, patients with

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hormone receptor positive tumors would benefit from endocrine therapy targeting ER; patients with HER2+ tumors are treated with the targeted therapy trastuzumab or the receptor tyrosine kinase inhibitors lapatanib; and TNBC patients lack approved targeted therapies and they are commonly treated with systemic chemotherapy [3].

Breast carcinogenesis is a complex process in which epithelial cells acquire genetic alterations within a permissive microenvironment that allows them to progress to a malignant neoplasm and subsequently metastasize to distant organs [4]. This tumor shows a great molecular and phenotypic heterogeneity, both at an inter- and intratumoral level, mainly governed by Darwinian selection driving tumor evolution [5-8]. Moreover, evidence demonstrate that subclonal populations of cancer cells may exist across different geographical regions within the same tumor, known as "spatial heterogeneity", and these populations may evolve over time differentiating the primary tumor from subsequent local or distant recurrence, known as "temporal heterogeneity" [9]. This high heterogeneity bears important implication in BC therapeutics, partially explaining the limited efficacy of targeted therapies in this tumor type.

Breast cancer metastasis is characterized by a multistep cascade which can be subdivided in different steps. (i) Detachment of tumor cells from the primary tumor, probably after undergoing epithelial-mesenchymal transition (EMT); (ii) Migration and infiltration of tumor cells into adjacent tissue; (iii) Transendothelial migration of tumor cells into vessels (known as intravasation), entering the blood as circulating tumor cells (CTCs); (iv) Survival of CTCs in the circulatory system; (v) Arrest of CTCs at secondary sites and extravasation as disseminated tumor cells (DTCs); (vi) proliferation of DTCs in distant tissues allowing colonization and growth of metastases [10].

Despite of the large number of tumor cells that are shed daily into circulation, experimental data suggest that only a tiny fraction of these cells are able to form macroscopic metastases, indicating that metastasis is a very inefficient process [11]. It is widely assumed that the reason for that inefficiency are the destruction of CTCs in the bloodstream by shear stress forces and immune attack, as well as a slow rate of extravasation and proliferation in the stroma at a secondary site [12]. Thus, only those cancer cells with the capability to survive in the bloodstream, adapt to the distant tissue and new microenvironment, and induce angiogenesis, will successfully seed metastases.

1.1.1 Escape from Primary Tumor and Infiltration of Neighboring Tissue

In the first steps of the metastasis breast tumor cells, either as individual cells or clusters, detach from the primary tumor invading into the surrounding tissue. In order to do so, carcinoma cells of epithelial origin loss cell polarity and modify cell-to-cell adhesion and cell-matrix adhesion escaping anoikis, a form of apoptosis that occurs in anchorage-dependent cells when they detach from the surrounding extracellular matrix (ECM) [13]. Some of the adhesion molecules involved in cell-to-cell interactions are cadherins, claudins, or plakoglobin. In particular, cadherins play an important role in mediating these interactions [14], as down-regulation of their expression is required to initiate metastatic outgrowth of BC [15]. The molecular and structural alterations needed for tumor cell invasion are mediated by a differentiation process known as epithelial-to-mesenchymal transition (EMT), consisting in a genetic reprograming of epithelial tumor cells by which they attained mesenchymal characteristics and characteristics resembling to those of cancer stem cells (CSCs) [16, 17]. Certain mesenchymal markers such as fibronectin, vimentin, and N-cadherin are activated during EMT, enhancing migration and favoring cell-to-stroma interactions [18, 19]. Under physiological conditions, EMT can be triggered by paracrine signaling of TGF-beta, WNT, plateletderived growth factors, or interleukin-6 (IL-6) [20]. Recent studies indicate that EMT is required for the dissemination of CTCs from breast tumors, however, EMT is not an on/off binary

switch, therefore resulting in hybrid or intermediate phenotypes [21]. It is now believed that these diverse phenotypes provide tumor cells the ability to adapt to the different microenvironments confronted along the metastatic process [22]. In addition to invasion of the surrounding tissue, the characteristics acquired by tumor cells trough EMT are also important for intravasation into the bloodstream and to induce the activation of proteases involved in the degradation of the ECM (including matrix metalloproteinases (MMPs)), thus EMT plays a major role in tumor progression [23]. However, there is evidence that EMT is not essential for metastasis [24, 25]. The importance of EMT in BC progression will be discussed in depth in a separate chapter of this book.

Once tumor cells are liberated from the originating tumor tissue and become motile, they can migrate either individually or collectively [26]. Main differences between these two forms of migration are the need for tumor cells to maintain stable cell-to-cell adhesion and multicellular coordinated movement in order to collectively migrate, whereas individual migration requires losing cell-to-cell adhesion [27]. While individual migration can be either of a mesenchymal or an amoeboid type of movement, collective migration may require leader cells exhibiting mesenchymal features and therefore mesenchymal migration [28]. Inner cells within these groups may retain an epithelial phenotype. In keeping with this, clusters of CTCs found in the blood of BC patients can show both epithelial- and mesenchymal-like phenotype [29]. An important feature of the collective migration is the protection of inner cells from insults such as immune attack and shear forces while in circulation.

In addition to this well described active migration, mobile tumor cells can also migrate through a passive mechanism by which they are "pushed" into blood circulation [12]. A hypothesis suggests that due to the effects of tumor growing, tumor cells can be shed into fragile and leaky tumor blood vessels that are being formed (by angiogenesis), accidentally ending up in the circulation [30]. An indication for this is the fact that a large fraction of epithelial CTCs found in the blood of patients with advanced BC are apoptotic [31], although it is difficult to determine whether apoptosis takes place before or after intravasation.

A critical factor for the progression of 'in situ' BC to metastatic breast cancer (MBC) is the interplay between tumor cells and tumor microenvironment [32]. This tumor microenvironment is comprised by many types of cells such as macrophages, fibroblast, endothelial cells and immune cells, together with the ECM, and it may be determinant on tumor progression. Indeed, in BC, evidences support a role for cancer associated fibroblasts (CAFs) aiding tumor cells migration [33, 34], as well as a role for tumor associated macrophages (TAMs) on promoting tumor growth, angiogenesis and immune response suppression [35].

1.1.2 Intravasation

Invading tumor cells have the ability to penetrate basement membranes and endothelial walls and lymphatic vessels, becoming CTCs, in order to spread to secondary sites for metastases formation. To complete this process, angiogenesis and/ or lymphangiogenesis are previously required [36], and tumor cells ought to disrupt the endothelial wall by mechanisms common to both the intravasation and extravasation processes [37, 38]. The vessels generated by tumor present weak cell-to-cell junctions which facilitate intravasation and the passive shedding of CTCs into the circulation [39]. However, active disruption of endothelial integrity increases the number of cells entering in blood or lymphatic vessels and therefore increasing metastasis [40]. In BC, dissemination can occur via the hematogenous and lymphatic systems. Lymphatic dissemination plays an important role in BC tumor cell spread [41]. Determining whether the cancer has spread to the regional lymph nodes is critical in staging a newly diagnosed patient, and the affectation of regional lymph nodes is considered to be a strong predictor of recurrences and survival [42]. Tumor cells disseminated to lymph nodes eventually exit via the efferent lymphatic vessels and make use of the hematogenous system that irrigates lymph

nodes to reach the blood circulation and disseminate to secondary organs.

1.1.3 Survival of CTCs in Circulation

As previously mentioned, during their transit in the circulation, CTCs encounter several obstacles such as shear forces, collisions with blood cells, attack of the immune system, and oxidative stress [43], which ultimately affect their survival and capacity to establish metastatic foci. The mechanical forces experienced by CTCs in the vasculature are a major interference with the survival of CTCs. Interestingly, experimental evidences show that CTCs which underwent EMT are more resistant against these insults than epithelial CTCs [44]. Moreover, CTCs have to resist anoikis and survive in the circulation in the absence of cell-to-matrix interactions which provide proliferation signals. In this regard, mesenchymal CTCs may have an advantage since they do not require these interactions for survival [10]. In support of this, it is the finding of CTCs with mesenchymal characteristics in the blood of patients with BC [29]. Also, some CTCs acquire anoikis resistance mechanisms such as the autocrine BCL2-dependent resistance mechanisms [45], or the activation of tropomyosin-related kinase B (TrkB), that enable cells to survive in suspension [13]. In addition, CTC clusters prevent tumor cells from anoikis by maintaining strong cell-to-cell interactions, promoting their survival in the circulation system [46, 47]. Another obstacle faced by CTCs in the blood circulation is the attack of the immune system, particularly from natural killer (NK) cells [48]. In order to evade the antitumoral surveillance, CTCs cooperate with platelets inducing their aggregation, what acts as a physical shield that protects CTCs [49, 50]. All these hurdles are responsible for the low survival rate of CTCs in the bloodstream, making metastasis a very inefficient process. It has been estimated that only <0.01% of CTCs with high metastatic potential give rise to distant metastasis [51], and that in BC, CTCs survive only a few hours in the circulation [52].

1.1.4 Extravasation to a Secondary Site to form Micrometastasis

CTCs must eventually extravasate and leave the circulation systems. In order to do so, CTCs slow down in small capillaries, attach to the endothelium lining of blood vessels, and finally undergo transendothelial migration [53]. Two main mechanisms for CTC extravasations have been proposed, (i) physical occlusion in capillaries of smaller diameter than CTCs, and (ii) cell adhesion to the endothelium in capillaries of higher diameter thanks to the expression of ligands and receptors on both CTCs and endothelial cells [10].

Recent evidences indicate that shear forces play an important role in this process, determining the place in the body where CTCs will extravasate from the blood [54]. Also platelet aggregation can aid CTCs by enhancing their adhesion to the vasculature, facilitating transmigration trough the endothelial barrier [50, 55]. In addition, BC CTCs increase the permeability of the vasculature by secreting soluble factors, such as TGF- β -induced Angiopoietin-like 4 [56], Angiopoietin-2 [57], and VEGF [58].

Upon extravasation in secondary sites, and in order to re-gain proliferation, DTCs must undergo a mesenchymal-epithelial transition (MET) reverting to an epithelial phenotype [59]. They must also scape immune surveillance, mainly mediated by cytotoxic T cells and natural killer (NK) cells [60]. But mandatorily, DTCs must adapt to the new microenvironment. Although the factors determining the adaptation of tumor cells in secondary sites are not well understood, the interactions between them and the microenvironment seem to be key [61]. The success of tumor cells in forming metastatic foci will be greatly determined by the microenvironmental niche, also known as metastatic niche. This metastatic niche represents a complex interplay among DTCs and resident cells (osteoblasts in the bone, hepatocytes in the liver, astrocytes in the brain, etc.), the ECM, and infiltrating cells such as immune cells [62]. But tumor cells have the capacity to establish a "premetastatic niche" [63] by which primary tumors release systemic signals (cytokines, exosomes, extracellular-matrixremodeling enzymes) that allow a more permissive and friendly microenvironment where DTCs can grow [43].

1.1.5 Breast Cancer Tropism and Dormancy

As already mentioned, in BC the initial dissemination of tumor cells is through the lymphatic system, reaching the sentinel lymph node from where they enter the blood circulation by exiting via the efferent lymphatic vessels. This tumor type metastasizes in an organ-specific manner preferentially to the bone and lungs and less frequently to other organs such as the liver and brain (95). Specifically, 47-60% of breast cancers metastasize to bone [64], 19–20% to the liver [65], 16–34% to the lung [66], and 10–16% to the brain [67]. This pattern of dissemination or organ tropism is mainly explained by circulation patterns that guide CTCs through the capillary bed where they arrest due to size restrictions of vessels whose diameter is too small to allow their passage [68]. Nevertheless, some CTCs are able to bypass this initial filter to reach other organs through the arterial circulation [43]. However, some BC CTCs may show preferences for specific tissues that favor their trapping through ligand-receptor interactions [69]. Indeed, several genes mediating preferential metastasis of breast tumor cells to bone, lung and brain have been identified [66, 70, 71].

The detection of metastases can occur many years and even decades after surgical resection of the primary tumor, indicating that CTCs extravasated in secondary organs as DTCs may remain in a dormant state. In BC, 20% of clinically disease-free patients relapse 7–25 years after mastectomy [72], suggesting the existence of a phenomena of early dissemination. Indeed, experimental evidences and clinical observations show that DTCs are detected in the bone marrow of people with no evidence of metastatic disease, in support of the idea that tumor cells can disseminate early, even before primary tumors become overtly invasive [61] or before the tumor is diagnosed. Thus, DTCs may give rise to occult micrometastases which mandatorily will undergo a growth restriction, and although the mechanisms of dormancy are still not clear, it is believed that tumor cells can survive in a quiescent state, in which they withdraw from the cell cycle, or by keeping a tight balance between cell proliferation at a slow rate and cell death [73]. The mechanisms and clinical implication of BC dormancy will be discussed in depth in a separate chapter of this book. It is only in the presence of the appropriate signals that DTCs may reenter into a proliferative state, colonize the secondary site, and eventually give rise to the formation of clinically relevant overt metastases [43].

1.2 CTCs Analyses, a Tool to Understand Breast Cancer Progression

Depending on the different subtypes, early-stage BC is amenable to curative tumor resection surgery. However, detection of early disease at the pre-symptomatic stages is very challenging. Once the local disease is detected, diagnosis based on small tumor samples or biopsies may result incomplete or incorrect given the high degree of heterogeneity of this tumor, which will consequently turn into a treatment which may be directed against targets not expressed throughout the entire tumor [74-76]. Likewise, in the metastatic setting, biopsies from metastatic lesion are usually not accessible, and therefore unable to guide therapy decision; and even when they are available, discordance between the primary tumor and recurrent metastasis in ER, PR and HER2 status has been shown [77, 78], with discordances of 6-40%, 21-41% and 1-43%, respectively [77]. Therefore, the study of CTCs through liquid biopsy techniques represents an optimal approach to address the heterogeneity of breast tumors, since they may represent the diverse molecular complexity of the tumors, and to limit the negative impact of heterogeneity in treatment selection. Moreover, CTC analysis will allow a more comprehensive understanding of the metastatic cascade, as they can originate

either from primary sites on route to metastatic niches, or from established metastases.

This is now possible thanks to the development of highly sensitive technologies able to capture these cells from a simple blood drawn from a cancer patient, in a serial and non-invasive manner. However, the detection of CTCs is not exempt of difficulties. CTCs are found in the bloodstream of patients but very rarely found in healthy individuals [79]. It has been estimated that there is 1-10 CTCs per mL of whole blood in patients with metastatic disease [80]. To date, more than 50 assays have been developed for the identification, enumeration, and even molecular characterization of CTCs. Apart from the very low frequency of CTCs, the main challenges that technologies face to detect tumor cells in bloodstream are to distinguish them from the large background of blood cells, and the phenotypical and molecular heterogeneity of CTCs. In this regard different strategies for CTCs identification and enrichment have been adopted. Generally speaking, the strategies developed can classify CTC enrichment technologies in two main groups, the ones that take advantage of the biological properties of the cells (surface marker expression), or the ones that take advantage of physical properties of the cell (i.e. size and density) [81], although a growing number of newer technologies combine both strategies. The first group comprises immunoaffinity-based methods that exploit the positive recognition of protein markers in the surface of the CTCs ("positive selection") by antibodies; being the most used marker the epithelial cell adhesion molecule (EpCAM). Among them, the CellSearch® system is the most frequently used for the isolation, enrichment and enumeration of CTCs in BC, and the only technology cleared by the U.S. FDA for clinical use in breast, prostate and colorectal cancer. All other technologies are available for clinical research. However, the CellSearch[®] system, and all technologies based on EpCAM recognition, present a major drawback, the downregulation or loss of expression of this epithelial marker during EMT [82], process that might be required for metastasis formation. The different technologies used for the isolation and enrichment of CTCs, together with the main advantages and disadvantages, will be discussed in a separate chapter.

Based on CTC counts provided by the CellSearch[®] system, CTCs are detectable in about 20-25% of patients with localized nonmetastatic BC at the time of diagnosis [83], while these figures reach and even surpass a 65% in patients with MBC [84]. These numbers put on evidence the challenge that represents to detect CTCs in the blood of BC patients, and in particular at an early stage cancer, which would mean the possibility to monitor and even prevent cancer relapse. Despite this, the clinical validity of CTCs in BC has been clearly demonstrated, and CTC levels have been proven to be a valuable tool to predict prognosis in BC patients. Thus, in MBC a count ≥ 5 CTCs per 7.5 mL of blood is associated with significantly inferior progressionfree survival (PFS) and overall survival (OS) [84]. Similarly, although with a lower threshold, in non-metastatic BC a CTC count ≥ 1 cells per 7.5 mL of blood is associated with decreased PFS and OS [83]. In addition to the prognostic value, CTC counts also enable prediction of treatment efficacy in patients with MBC [85-87]. Therefore, CTC enumeration is an effective prognostic and predictive biomarker, allowing early detection of metastasis development and monitoring of therapies efficacy. However, despite these clinical evidences, CTCs have not been included yet into the clinical guidelines, and their clinical utility, meaning the capacity to guide therapy decision and improve patient outcomes, remains to be determined in clinical trials. This topic will be further discussed across different chapters of the book.

In addition to enumeration, CTCs isolated from the blood of cancer patients can be characterized at the molecular and genomic level through the use of methods based on DNA, RNA, and protein analysis, either as a pool of cells or at single cell level. This characterization would enable the possibility to identify therapeutic targets and resistance mechanisms to targeted therapies [88]. Furthermore, it would bring the opportunity to adapt therapeutic strategies and to improve treatment selection, which would lastly translate into individualized treatments and a personalized medicine [89]. Interestingly, the characterization of CTCs at protein, RNA and DNA level is already providing relevant information for the identification of therapeutic targets and resistance mechanisms in BC [90, 91].

In summary, CTC analyses have the potential to elucidate the dynamics of the progression from localized BC to MBCs, changing our understanding about the metastatic process, and identifying the characteristics of the cells with the capacity to initiate cancer metastasis. Moreover, the analysis of CTCs bears a great potential to improve the management of BC patients, changing the landscape of BC treatment and even preventing the progression towards the metastatic disease. Specifically, it will be instrumental for the identification of new therapeutic targets in order to prevent metastatic recurrence, and to monitor treatment and understand the mechanisms of drug resistance, hereby representing a key tool to achieve a more personalize management of breast cancer patients.

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2

Epithelial-Mesenchymal Plasticity in Circulating Tumor Cells, the Precursors of Metastasis

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Abstract

Circulating tumor cells offer an unprecedented window into the metastatic cascade, and to some extent can be considered as intermediates in the process of metastasis. They exhibit dynamic oscillations in epithelial to mesenchymal plasticity and provide important opportunities for prognosis, therapy response monitoring, and targeting of metastatic disease. In this manuscript, we review the involvement of epithelial-mesenchymal plasticity in the early steps of metastasis and what we have learned about its

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Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA e-mail: maheswaran@helix.mgh.harvard.edu contribution to genomic instability and genetic diversity, tumor progression and therapeutic responses using cell culture, mouse models and circulating tumor cells enriched from patients.

Keywords

Breast cancer · Circulating tumor cells (CTCs) · Epithelial-mesenchymal plasticity (EMP) · Metastasis

2.1 Defining Epithelial-Mesenchymal Plasticity

Cancer metastasis, the major cause of patient mortality, is a complex multi-step process in which tumor cells become invasive, intravasate into the blood, survive in the circulation, extravasate out of the blood stream, and proliferate at the distal sites. During the early steps of metastasis, tumor cells lose apico-basal polarity through disruption of cell-cell interactions and cytoskeletal remodeling to support invasion [1]. These changes are reminiscent of the normal physiological process, epithelial to mesenchymal transition (EMT), that is required for gastrulation, neural crest cell migration, heart morphogenesis, organogenesis, and wound healing [1–7]. Utilization of EMT by cancer

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cells to migrate, invade, and survive when nonadherent provides an attractive model to understand the critical steps involved in the initiation of metastasis. The process of canonical EMT in cancer cells is generally attributed to epigenetic changes that are thought to be largely reversible upon removal of EMT stimuli, resulting in the reversion of this phenotype through mesenchymal-epithelial transition (MET). It is generally accepted that MET plays an important role in successful completion of the metastatic cascade with epithelial-like tumor formation [1, 7–11], and recently emerged controversies [12, 13] have been addressed [14, 15].

We will refer to these lineage switches as epithelial-mesenchymal plasticity (EMP) from this point forward to reflect the extensive bidirectional plasticity of the process. EMP phenotypes have been observed in cell culture and in mouse tumor models of breast, lung, prostate, pancreatic, colorectal, and ovarian cancers [16–18]. Detection of EMP in patient-derived tumor tissue specimens, however, has been complicated by the presence of stromal cells which express high levels of mesenchymal markers. As such, despite the dramatic invasive and tumorigenic phenotypes observed in mouse xenografts expressing EMP-regulating master transcriptional factors, Snail, Twist, and Slug among others, the direct observation of EMP in the metastasis of human epithelial cancers has remained elusive. Recently, tumor cells at various stages of EMP were detected in the blood of breast cancer patients, suggesting that EMP is not bimodal but is a continuous process [19]. These circulating tumor cells (CTCs) are extremely rare and are the putative precursors of metastasis. Therefore, defining EMP as a single dramatic transition between two states may be an oversimplification and may limit the study of EMP in some circumstances [7]. In this chapter, we will review the process of EMP, the evidence for EMP in clinical samples, its contribution to breast cancer dissemination (with a focus on the metastatic intermediates, CTCs), and the therapeutic implications associated with this process.

2.1.1 Epithelial-Mesenchymal Plasticity to Model the Early Steps of Metastasis

During EMP, epithelial cells within the primary tumor switch lineage to take on a more mesenchymal phenotype [1, 20], which is associated with morphological changes and molecular reprogramming [5, 21]. This consists of a series of sequential processes: the loss of apico-basal polarity due to cytoskeletal and junctional remodeling, increased cell migration as the result of decreased cell-cell adhesion and increased motility (sometimes at the cost of proliferation), and the acquisition of invasive properties such as passage through a basement membrane [1]. The basement membrane between the epithelia and nearby blood vessels is the first barrier encountered by invading cells [22]. Invasion requires breach of the basement membrane, then breakdown of the extracellular matrix in the stroma by proteases such as matrix metalloproteinases [23]. EMP regulates expression of many of the genes required for this breach of the basement membrane and matrix. Upon arrival at the secondary site, MET then proceeds in the reverse order, with increased polarity and cell-cell adhesion leading to decreased cell migration and an epithelial phenotype associated with increased proliferation. The steps of this process are highlighted in Fig. 2.1.

2.1.2 Inducers and Effectors of EMP

EMP in both development and cancer is induced and maintained by a variety of signals: (i) extracellular signals, (ii) master transcription factors, and (iii) post-transcriptional regulators. Extracellular signals regulating EMP consist of peptide growth factors (e.g. FGF, EGF, HGF, TGF β), cytokines, differentiation factors (Wnt, Notch, SHH, NF κ B pathways, RAS/receptor tyrosine kinases), and hormones secreted by the cancer cells themselves as well as the supporting cells in the tumor microenvironment [1, 7, 24– 30]. Additionally, hypoxia and extracellular com-



Bone Micro-Metastasis

Bone Metastasis

Fig. 2.1 Metastatic cascade highlighting CTC and EMP characteristics. A small proportion of carcinoma cells exhibit epithelial mesenchymal plasticity, resulting in hybrid (E/M) phenotype rather than a distinctly mesenchymal phenotypes (M). These mesenchymally shifted cells are associated with loss of the basement membrane and migration / invasion into the tumor microenvironment, where they can remain dormant. Epithelial change in these cells is likely to underpin local recurrence, allowing a new colony to form. A higher proportion of mesenchymally shifted (E/M) cells is found in the vasculature as circulating tumor cells

ponents such as collagen also can induce EMP [1, 25–29, 31–33]. These extracellular signals are transduced to transcription factors that regulate the expression changes required to elicit epithelial-mesenchymal state change. The master transcriptional regulators of EMP include Snail/ Slug, Twist, and members of the Zeb transcription factor family [1, 34]. EMP is also regulated by post-transcriptional processes including ubiq-

(CTCs), indicating their increased capacity for intravasation and survival in the vasculature. A full spectrum of epithelial (E) to mesenchymal phenotypes is seen in the blood however, the hybrid phenotype dominates. CTC clusters containing cells at different stages of the EMP spectrum, and also normal immune cells and in some cases, tumor stromal cells, are also seen and have a higher prognostic value and a higher patho-biological potential. Dormant single cells / micrometastatic deposits can be seen in the bone marrow (depicted) or other metastatic sites. MET results in slightly altered gene expression profiles (E^M)

uitination, alternative splicing, and miRNAs that regulate protein translation, the most well characterized being the miR-200 family which modulates the expression of the (ZEB) proteins [1, 7, 34–36].

Along with a multitude of additional modulators and chromatin modifiers, these regulators coordinate the expression of proteins that maintain the epithelial state, apico-basal polarity, and cell-cell adhesion, including Crumbs, PAR, Scribble, E-cadherin, α -catenin, γ -catenin/plakoglobin, and claudin. They also regulate proteins defining the mesenchymal state, cellular motility, and invasiveness, including N-cadherin, vimentin, and fibronectin [1, 7, 19, 34]. Together, these many inputs create a broad and often redundant signaling network to induce and maintain these states of plasticity in tumor cells [1, 2, 5, 25–27, 37–41].

2.1.3 EMP in Cancer Stem Cells and Drug Resistance

In addition to being involved in promoting metastasis, EMP has also been implicated in contributing to the maintenance of cancer stem cells (CSC). Like CSCs, cells undergoing EMP can survive under adverse conditions and exhibit resistance to chemotherapeutic interventions, although they do not necessarily self-renew [42]. Cells undergoing EMP coincidently acquire many CSC markers. In breast cancer, the presence of mesenchymal markers correlated with the presence of CSC markers including ALDH1, NANOG, OCT-4, and CD44 [43]. Double knockdown of the cancer stemness markers NANOG and OCT-4 reversed EMT in lung adenocarcinoma, while induction of these genes promoted EMT in breast cancer [43]. Similarly, knockdown of the OVOL2 transcription factor in nasopharyngeal carcinoma cells decreased both EMT and stemness [44]. Upregulation of CSC markers and the appearance of a CSC phenotype during EMP has been observed in cell lines, mouse models, and patient samples [43, 45-48]. However, EMP is not always associated with the appearance of CSClike properties. CSCs consist of both mesenchymal and epithelial phenotypes under different contexts, while EMT is often associated with a more mesenchymal state [49]. Further, CSCs represent a minor population of all tumor cells, whereas EMP occurs in a much larger fraction of tumor cells suggesting that additional criteria are involved in defining the functional characteristics of CSCs [50]. Further studies are required to better define the relationship between EMP and CSCs, specifically whether they represent a common phenomenon and if they are both induced and maintained through the same inducers and pathways.

Across several cancer types, the mesenchymal state is associated with increased drug resistance while the epithelial state is associated with increased sensitivity [34, 51-53]. In a mouse model of breast cancer, cells forced to revert to the epithelial state lost CSC markers and were increasingly sensitive to doxorubicin, paclitaxel, proteasome inhibitors, and MAPK/ EGFR inhibitors [54, 55]. Further, neoadjuvant chemotherapy in breast cancer has been shown to be ineffective against CTCs in the EMP state [56, 57]. EMP signatures were also found to be associated with treatment response and resistance in non-small cell lung carcinoma, pancreatic, breast, and ovarian cancer [5, 12, 13, 30, 58–60]. The mechanistic aspects of EMP thought to confer drug resistance are similar to those in CSCs and include elevated expression of antiapoptotic proteins and drug efflux transporters and immunosuppression through the activities of EMP master transcription factors [50, 61].

2.1.4 Significance of EMP in Nonepithelial Cancers

While EMP is important for tumors of epithelial origin to migrate to the metastatic site, tumors of non-epithelial origin – leukemias, lymphomas, myelomas, sarcomas, and brain and spinal cord cancers – do not necessarily encounter these barriers. For some non-epithelial cancers, such as glioblastoma, markers of EMP are still induced by microglia and macrophages via NFkB and support invasiveness [30, 62, 63]. Further, of the four glioblastoma subtypes, the mesenchymal subtype is the most aggressive and radioresistant [64–66]. In sarcomas such as osteosarcoma and

rhabdomyosarcoma, where the cell of origin is already highly mesenchymal, further upregulation of the EMP transcription factor ZEB1 has been observed compared to normal tissue, and SNAIL expression was associated with poorer overall survival [67–69]. Higher expression of epithelial E-cadherin is also associated with improved survival in bone and soft tissue sarcomas [70].

2.1.5 Contribution of EMP to Genomic Instability and Genetic Diversity

A series of studies published several years ago showed that mitosis during Drosophila and Xenopus embryogenesis is actively inhibited in cells undergoing gastrulation. Premature induction of proliferation before the completion of gastrulation in cells undergoing EMP results in extensive developmental abnormalities [71–74]. Recent studies show that this embryonic process is exploited by the tumor cells to drive genomic instability and diversity [75] – changes that can have profound consequences on tumor progression and drug responses. Although transitioning of epithelial cells to a mesenchymal state is reversible upon removal of the EMP inducers, the induced abnormalities in ploidy and genomic heterogeneity are heritable. The mechanistic clue to this incompatibility came from detailed proteomic analysis, which revealed that several nuclear envelope proteins are suppressed as epithelial cells transition to a mesenchymal state. Nuclear envelope proteins, in addition to providing the structural framework of the nucleus and selectively modulating the passage of molecules between the cytoplasm and the nucleoplasm, also play critical roles in orchestrating proper mitosis. Therefore, while the suppression of nuclear envelope proteins reduces the rigidity of the nucleus to facilitate EMP-associated migration and invasion, the requirement of these proteins for mitosis [76, 77] might also render their decrease during EMP incompatible with simultaneous proliferation. Subsequent studies show that clonal epithelial populations spontaneously generate mesenchymal variants, which can revert to an epithelial phenotype [78] contributing to chromosome instability and the selection of robust variants capable of forming metastatic tumors. Disruption of tissue architecture associated with this cell fate switch has also been implicated in maintaining the fidelity of chromosome segregation [79].

2.1.6 Mouse Models of EMP

The inherent plasticity of EMP makes unequivocal determination of the lineage for a given cell difficult. Most studies evaluating the role of EMP in disease progression in vivo have relied on xenograft mouse models and cultured cells. Experimental induction of EMP in cancer cells led to an increase in metastasis, and knockdown of EMP or premature induction of MET reduced metastasis [47, 80–82]. Interestingly, expression of the EMT-inducing homeobox transcription factor, Prrx1, led to EMT phenotypes in cultured cancer cells [47]. However, loss of Prrx1 in cultured cells was required for efficient metastasis upon tail vein injection or orthotopic tumor formation in mice [47]. Other studies utilized mouse models with intrinsic EMP reporters and gain-offunction or loss-of-function of EMP master transcription factors [13, 20]. In skin-specific Twist1-inducible mice, Twist1 induction caused higher rates of squamous cell cancer development upon treatment with a carcinogen [83]. Reversal of this Twist1 induction upon tumor cell dissemination significantly increased metastasis. Together with the Prrx1 data, this result strongly supports a role for MET in metastatic outgrowth [83]. Single-cell lineage tracing with reporter genes irreversibly activated by lineage-specific promoters have been used to query the fate of the cells experiencing EMP. Reporter genes thus activated by epithelial/mesenchymal promoters have been used to track EMP in mouse models and monitor the change in cellular states during the course of metastasis and tumor progression [84]. Breast cancer models have found that a small fraction of primary and metastatic tumor cells undergo EMT [12]. Conversely, pancreatic cancer models showed about half of tumor cells had undergone EMT, rarely occurring in premalignant lesions [85]. However, given the complex signaling networks involved in promoting EMP, it is difficult to reach concrete conclusions based on studies that rely on a single marker in a given model, particularly in the context of the EMP hybrid phenotype, where the degree of induction may be less strong.

2.1.7 Detecting EMP in Clinical Samples

Although lineage tracing in humans is not possible and acquisition of serial samples is quite difficult, evaluation of epithelial and mesenchymal markers in patient-derived tissue provides a snapshot EMP of in the clinical setting. Immunohistochemistry (IHC) of human breast cancer samples with mesenchymal markers such as vimentin, N-cadherin, cell cycle, and tumor specific markers such as HER2, showed evidence for EMT in triple negative and basal-like tumors but not in invasive lobular carcinomas [86, 87]. RNA in situ hybridization (RNA-ISH) using multiple probes to detect both epithelial and mesenchymal transcripts in the same samples delineated the ratios of epithelial and mesenchymal tumor cell populations at the single cell level in the primary tumors and draining lymph nodes of human breast cancer specimens [19]. While most tumor cells exhibited an epithelial phenotype, triple negative breast cancer was enriched for cells with mesenchymal markers, and all subtypes contained rare cells with combined epithelial and mesenchymal staining [19]. RNA-ISH analysis was also performed on CTCs from breast cancer patients, where it performed significantly better at detecting mesenchymal cells compared to standard cytokeratin approaches (discussed in more detail below) [19]. CTCs provide a noninvasive tool to monitor EMP in real time as

patients progress through therapeutic interventions.

2.2 EMP in Circulating Tumor Cells

During metastasis, CTCs – the putative metastatic precursors – travel through the blood. Although the majority of CTCs are destroyed in the blood through apoptosis, the remaining viable cells reach and reside within distal sites in a dormant state until they adjust to the new microenvironment and eventually proliferate. The relative accessibility of CTCs in the peripheral blood provides real time sampling of tumor cells to interrogate the contribution of EMP to metastasis and drug responsiveness [30].

Studies of CTCs provide some of the best evidence for the involvement of EMP in promoting metastasis. Mesenchymal markers have been observed in CTCs from patients with glioblastoma, breast, liver, nasopharyngeal, colon, gastric, bladder, pancreatic, and non-small cell lung cancers [7, 19, 30, 54, 56, 80, 88–97]. A summary of EMP studies in breast cancer CTCs is shown in Table 2.1. These studies showed that CTCs are a heterogeneous population and, as predicted, exhibit more mesenchymal characteristics compared with the cells in the primary or metastatic tumors. A large fraction of individual CTCs was also found to express both epithelial and mesenchymal markers, suggesting that plasticity is a common component of the metastatic phenotype [7, 19, 30, 56, 80, 91–96]. Lineage tracing experiments in animal models will be required to explore the stage of tissue residence or circulation at which CTCs undergo both EMT and MET [7].

2.2.1 Hybrid-EMP and CTC Clusters

Recent studies regarding EMP in CTCs address a longstanding dispute in the field: whether EMP should be defined as a binary process with epithelial and mesenchymal endpoints as observed in most non-disease cases (with notable exceptions

MT markers and 1 other factors) References	[98] [98] [esponders	[66]	[100]	irculating [101] imerous in	both in EBC [102]	ith disease [103]	ooth in EBC [104]	ddCTC) [105] lymph node	[106]	[107]	[108]	patients with [109] ascription	(continued)
Correlation between EN clinical parameters (and	CTC ^{EMT+} higher in therapy non-1	CTCEMT+ detected	CTCEMT+ patients \PFS	CTC ^{EMT+} and EMT+ Ci Tumor Emboli more nu MBC patients	EMT markers detected and MBC patients	CTC ^{EMT+} correlation wi	EMT marker detected t and MBC patients	dedifferentiated CTC (correlate with negative status	No correlation found	CTC ^{EMT+} detected	No correlation found	L PFS/early relapse in J high levels of EMT trai factors	
Identification/ epithelial markers	EpCAM, MUC1, HER2	CKs	CKs	CKs	CK19 MGB1 HER2	CKs	CK19 MGB1 HER2		EpCAM MUC1 HER2	CKs	EpCAM MUC1 HER2	1	
CTC Detection/ characterization method	RT PCR	IF	RT PCR	IF	PCR-liquid bead array hybridization	RT PCR	RT-PCR	RT PCR	RT PCR	RT PCR	RT PCR	RT PCR	
Method of CTC enrichment	AdnaTest BreastCancer	CellSearch EpCAM-isolation	Epcam positive CELLection beads	Ficoll density gradient/ CD45 negative Dynal CELLection beads	EpCAM positive CELLection beads	EpCAM positive CELLection Dynabeads	EpCAM positive CELLection beads	AdnaTest EMT-1/Stem Cell	AdnaTest Breast Cancer	CD45/CD326 magnetic beads depletion	AdnaTest Breast Cancer	CD34 depletion/ Ficoll density gradient/CD45 magnetic beads depletion	
Nr of patients/ Breast cancer type	39 M ₁	16 M1	55 M ₁	25 M ₀ 25 M ₁	64 M0 20 M1	92 M ₀ +M ₁	66 M ₀ 26 M ₁	$61 \mathrm{M}_0$	$130 M_0$	28 M ₁	$502 \mathrm{M}_0$	21 M ₁	
EMT/CSC markers (+ other markers co-analyzed)	Twist1, PI3Kα, Akt2, ALDH1	Vim, E-cad, N-cad, O-cad, CD133	Vim, FN	Vim, Twist1	Twist	Vim, FN, ALDH1	Twist	Twistl, PI3K-α, Akt2, ALDH1, Bmi1, CD44	Twist1, PI3K-α, Akt2, ALDH1	Twist1, Snail, Zeb1, TG2	Twist1, PI3K-α, Akt2, ALDH1	Twist1, Snail, Slug	

Table 2.1 (continued)	(p					
EMT/CSC markers (+ other markers co-analyzed)	Nr of patients/ Breast cancer type	Method of CTC enrichment	CTC Detection/ characterization method	Identification/ epithelial markers	Correlation between EMT markers and clinical parameters (and other factors)	References
Twist1, Snail, Slug, Zeb1, FoxC2	52 M ₀	CellSearch, AdmaTest Breast Cancer Ficoll density gradient + CD45 magnetic bead depletion	RT PCR	_	CTC ^{EMT+} resist neoadjuvant therapies	[110]
CD44, CD47, MET	4 M ₁	CellSearch RosetteSep hematopoietic cells depletion/ FACS EpCAM-CD45	FACS	EpCAM	CTCs ^{CD44MET+CD47+} increase with disease progression and correlate with patient metastatic burden and Joverall survival	[11]
N-cad,FN,PAII,E-cad	41 M ₁	Herringbone-chip EpCAM, HER2, EGFR positive selection	RNA-ISH	CKs, EpCAM,	CTC ^{M+} associate with disease progression CTC ^{M+} observed as both single cells and multicellular clusters Reversible shifts between CTC ^{E+} /CTC ^{M+} accompanied each cycle of response to therapy.	[61]
Twist1, Snail, Slug, Zeb1	M ₀	RosetteSep selection kit CD45 negative selection	RT PCR	CK19	CTC ^{EMT+} associate with high tumor grade and expression of MMP-1 in tumors.	[112]
Vim, Snail, Twistl, Slug, uPAR	117 M ₀	Density gradient centrifugation/ CD45 magnetic particle depletion	RT PCR	CK19 MGB1 HER2	CTC ^{EMT+} associate with Lymph node involvement	[113]
Vim	98 M ₀	Density gradient centrifugation/ CD45 magnetic particle depletion	RT-PCR	CK19 MGB1 HER2	CTC ^{EMT+} predominantly detected in patients who died during follow up. CTC ^{EMT+} more frequent in patients with low E-cad in the primary tumor.	[114]
Twist1, ALDH1	$\begin{array}{c} 80 \ M_0 \\ 50 \ M_1 \end{array}$	Ficoll density gradient+cytospins	IF	CKs	CTC ALDHINigh/NuclearTwist more frequently detected in MBC	[115]
Vim, Slug, EGFR	78 M ₀	Carcinoma Cell Enrichment and Detection kit, MACS technology CK magnetic beads positive selection	IF	CKs	CTC ^{EMT+} correlate with tumor size	[116]
Ratio vim/CKs	61 M ₁	CellSearch	IF	CKs	CTC ^{CK low} correlate with TNBC and shorter OS	[117]

Surface vimentin Twist1, Snail, FoxC2, E-cad	58 M ₁	EasySep TM Human CD45 Depletion Kit + positive collection with surface vimentin ab magnetic beads	H	EpCAM	CTC ^{EMT+} count higher in treatment non-responders (association with disease progression)	[118]
Plastin 3	594 M ₀ +M ₁	FICOLL density gradient	RT-PCR	I	Higher Pls3 levels in TNBC Pls3-positive patients have poorer OS/ DFS	[119]
Vim	221 M ₀ +M ₁	1	RT-PCR	EpCAM CK19 HER2	CTC ^{EMT+} detected in all stages CTC count decrease after treatment	[120]
Vim, Twist1	18 M ₀ +M ₁ (other type of tissues analyzed)	CD45 depletion/ CanPatrol (filtration system)/IF-FISH CD45	RNA-IF	EpCAM, CK8/18/19	CTC ^{EMT+} higher in MBC patients	[94]
Vim, Tissue Factor	22 M ₁	ScreenCell filtration device	IF	CKs	Detection of Vim+/Tissue Factor+ CTCs	[121]
N-Cad, CD44, CD146, E-cad	47 M ₁	CD45 MicroBeads depletion/ DEPArray sorting CD45 negative cells	II	EpCAM	CTC ^{EMT+} associate with shorter PFS/ OS	[122]
Vim (55 breast cancer associated genes)	147 M ₁	AdnaTest BreastCancer	RT PCR	EpCAM MUC1 HER2	Vimentin increased in CTC-enriched samples	[123]
EpCAM	32 M ₁	Parallel multi-orifice flow fractionation chip (p-MOFF system)/ IF CD45-CK	IF	EpCAM, CK 7, 8	ND	[124]
Twist1	45 M ₁ (before and after aromatase inhibitors)	CellSearch Profile Kit (EpCAM- positve Magnetic beads selection)	RT-PCR	CK8 within the 8 gene signature	96 genes analyzed, 8 gene-signature (including Twist1) for non-responders	[125]
Vim	22 M1 (before and after eribulin treatment)	microfluidic chip	H	CKs	CTC ^{EMT+} more frequently observed in TNBC Patients with more total CTCs and more CTC ^{EMT+} have a shorter PFS Total CTC count increase with disease progression	[126]
						(continued)

	fT markers and other factors) References	TC ^{EMT+} [127] motherapy but	tus defined in [128] d epithelial- ociate with t and larger relate higher
	Correlation between EM clinical parameters (and	PFS↓ in patients with C Total CTC↓ during chen proportion of CTC ^{EMT+} ↑	Different EMT CTC stat the cohort. Mesenchymal CTCs and mesenchymal CTCs asso lymph node involvemen tumor size. Mesenchymal CTCs cor risk of death.
	Identification/ epithelial markers	EpCAM CK8/18/19	CK19, MGB1, HER2
	CTC Detection/ characterization method	RNA-ISH	RT-PCR
	Method of CTC enrichment	CanPatrol CTC filtration system	Density gradient centrifugation/ CD45 magnetic particle depletion
(1	Nr of patients/ Breast cancer type	108 M ₁	83 M ₀
Table 2.1 (continued	EMT/CSC markers (+ other markers co-analyzed)	Vim, Twist1	E-cad, N-cad, Vim, Pls3, CD44, NANOG, ALDH1, OCT-4, CD133, plakoglobin

Vim vimentin, FN fibronectin, CKs cytokeratins, Cad cadherin, MGBI mammaglobin A, Pls3 plastin3, Ab antibody, Mo non metastatic breast cancers, M_I metastatic breast cancers, IF immunofluorescence, ISH in situ hybridization, OS overall survival, PFS progression-free survival, RFS relapse free survival, DFS disease-free survival R. Burr et al.

of cohort migration as outlined below), or whether EMP is a spectrum phenotype with potentially stable higher-plasticity manifestations along the continuum from epithelial to mesenchymal phenotype [1, 53]. Highly-plastic cells with both epithelial and mesenchymal phenotypes (hereafter referred to as hybrid-EMP) are observed in many CTCs as well as in vivo; in fact, hybrid-EMP CTCs are more commonly observed than fully mesenchymal cells in many studies [8, 129]. Two recent studies of mouse pancreatic ductal adenocarcinoma (PDAC) and skin squamous cell cancer showed that these hybrid-EMP cells are more plastic than either epithelial or mesenchymal cells, with a higher ability to interconvert among the types in culture [8, 129]. Epigenetic, transcriptional, and posttranscriptional mechanisms were identified as regulating this interconversion, suggesting further study is needed to devise a unified mechanism for hybrid-EMP plasticity or to identify disease-specific mechanisms [8, 129]. Based on this data, we and others define EMP as a spectrum of phenotypes with highly plastic interconversion among the different states, with increased appreciation that the location of any particular cell along this continuum has important implications for both cancer and development [1, 7, 8]129–137]. However, it is important to note that while the existence and importance of hybrid-EMP is accepted, it is still unclear whether it represents an intermediate phase during EMP or a final state, and even whether the same signaling pathways at work during EMP are also responsible for hybrid-EMP [7, 8].

Practically, the label hybrid-EMP is assigned to varied states, which include cells that downregulate epithelial markers but do not upregulate the full complement of mesenchymal markers, as well as cells expressing both epithelial and mesenchymal markers [8, 129]. For example, cells with upregulation of mesenchymal processes such as loss of polarity and increased motility and invasion but without loss of cell-cell adhesion or cell individualization. Indeed, although individual cell migration is a hallmark of EMT,

recent studies have highlighted the presence of multicellular CTC clusters (up to 100 cells) in the circulation of patients with advanced cancers such as inflammatory breast cancer, and cohort migration is accepted as a frequent mode of invasion [1, 51, 138–147]. Clusters are more effective at colonizing secondary sites than single CTCs and correlate with a worse prognosis [7, 19, 80, 139]. Importantly, there is an association between CTC expression of mesenchymal markers and cluster formation. Many clusters are coated in platelets, which are a source of TGF β and may help induce or maintain mesenchymal characteristics [30, 148]. These clusters necessarily maintain cell-cell contacts and some epithelial-like expression (notably the desmosomal protein plakoglobin), suggesting that they exhibit the hybrid-EMP phenotype described above [8, 51, 80, 149, 150]. Indeed, tumor spheres of hybrid-EMP mouse prostate cancer cells exhibited collective cell migration and cluster delamination while fully mesenchymal spheres only showed single-cell invasion [8]. It is not clear whether hybrid-EMP clusters are composed of a homogenous population of hybrid-EMP CTCs versus a mixed population of epithelial CTCs and mesenchymal CTCs [80]. However it is important to note that lineage tracing and tumor transplantation experiments show that CTC clusters do not form in the bloodstream through aggregation of single CTCs, but originate from polyclonal primary tumors, suggesting that the hybrid-EMP phenotype is established before invasion into the circulation [139, 151]. Although these clusters may seem impossibly large for invasion or extravasation through the blood vessel into the secondary tissue, studies have shown that CTC clusters can traverse the capillaries of Zebrafish by rapid reorganization into single-file chains [140]. Finally, although cohort migration is highlighted in the study of cancer CTCs, it should be noted that similar modes of invasion occur during development, wound healing, and mammary reorganization in some species, underscoring the fact that this hybrid-EMP phenotype is not restricted to the cancer environment [1].

2.2.2 Role of EMP in CTCs During Progression and Therapeutic Response

The implications of hybrid-EMP phenotypes on tumor histology and prognosis are significant. Hybrid-EMP cells are detected in both primary and metastatic tumors and are particularly prevalent in individual and clustered CTC populations as noted above [19]. Single-cell evaluation of both EMP markers and tumor-specific markers (such as HER2) in breast cancer confirm that these hybrid-EMP cells are tumor-derived [19]. Mesenchymal mouse PDAC tumors were poorly differentiated while hybrid-EMP tumors were moderately to well-differentiated [8]. Similar results are observed in human poorly differentiated quasi-mesenchymal, squamous, or basallike PDAC tumors versus well-differentiated classical/exocrine-like, classical, or pancreatic progenitor/ADEX tumors [8, 152–154]. The proportion of breast cancer CTCs with fully epithelial, predominantly mesenchymal, or hybrid-EMP seems to be dependent on tumor type and stage, consistent with data for primary and metastatic tumor cells. Pre-invasive ductal carcinoma in situ (DCIS) lesions exhibit exclusively epithelial phenotypes, while invasive breast cancers contain rare hybrid-EMP cells, suggesting incomplete MET [19]. Further, CTCs from patients with lobular type (ER+/PR+) cancers were predominantly epithelial while CTCs from patients with HER2+ or triple negative breast cancers were predominantly mesenchymal [19].

Studies show that EMP phenotypes in CTCs indicate poor prognosis and resistance to therapy. Hybrid-EMP mouse skin cancer cells produced more metastasis after tail vein injection than fully mesenchymal cells [129]. In humans, EMP CTCs confer poor prognosis in breast, prostate, liver, colorectal, head and neck, pancreatic, endometrial, and lung cancers [155]. Hybrid-EMP cells are more anoikis-resistant and drug-resistant [53, 156], giving them a better chance of metastatic colonization. Although the signaling pathways mediating anoikis resistance are not fully understood, EMP markers such as TGF β , Twist, Snail, and miR200 have also been

shown to have effects on survival in circulation [157, 158]. This is particularly significant to breast cancer treatment, as apoptosis is the main inducer of regression in systemic therapy and resistance of disseminated tumor cells to apoptosis is correlated with worse prognosis [159]. EMP CTCs were also associated with chemotherapy or radiotherapy resistance in ovarian, breast, and colorectal cancer [109, 160, 161]. Interestingly, when one breast cancer patient was followed longitudinally, mesenchymal-CTCs decreased with therapy response and then increased upon development of resistance, a phenomenon that was observed over two successive rounds of treatment. This increase in mesenchymal-CTCs was accompanied by the appearance of CTC-clusters [19].

2.2.3 The Influence of EMP on CTC Isolation Technologies

The plasticity of mixed epithelial and mesenchymal CTC phenotypes in the blood has been highly consequential in defining the capture efficiency of antibody-based CTC isolation approaches that rely on the expression of the epithelial marker EpCAM on the surface of tumor cells. The only FDA approved technique for in vitro diagnostic use, CellSearch®, (Veridex, Menarini Silicon Biosystems) uses immunomagnetic beads coated with antibodies against EpCAM. Other CTC isolation modalities rely on physical characteristics such as size, density, deformability, and charge. However, because CTCs are highly heterogeneous and many CTCs exhibit a hybrid-EMP or fully mesenchymal phenotype, enrichment by a single epithelial surface marker or physical characteristic may not be sufficient to capture the full array of CTCs in the blood [7]. To overcome this limitation, techniques using multiple antibodies that mark epithelial and mesenchymal states (e.g. a combination of EpCAM, cytokeratin, and vimentin) and tumor-specific cell surface markers including HER2 and EGFR have been more effective in sampling the different populations of CTCs circulating in the blood [19]. However, these "double positive" isolation technologies
still fail to enrich for hybrid-EMP CTCs that express neither epithelial nor mesenchymal commonly examined markers [8]. Negative depletion of leukocytes with antibodies directed against white blood cells provides an efficient method to overcome the limitations posed by positive selection. These technologies rely on a permissive size-based separation to eliminate red blood cells followed by immunomagnetic depletion of white blood cells with CD45 antibodies [162–165]. They are considered negative selection because they enrich for CTCs based on known properties of the other cellular components of the blood, rather than making assumptions about CTC phenotypes that could bias the population of CTCs after isolation. The capability of each technique to isolate epithelial, mesenchymal, and hybrid-EMP CTCs is shown in Table 2.2. They each have their strengths and weaknesses, which are important to appreciate, however to date there in no universally accepted preferred method that allows comprehensive capture of all CTCs.

		Detection of Epithelial (E),				
		Mesenchymal (M), or Hybrid				
Method	Name	(H) CTCs	References			
Physical separation (1997)						
Size based filtration/microfluidics	Microcavity array (MCA); FAST disc; CellSieve	$\mathbf{E} = \mathbf{M} = \mathbf{H}$	[166–168]			
Density based centrifugation	Ficoll; OncoQuick	E = M = H	[169]			
Size and deformability	ISET®; Celsee	M > H > E	[170, 171]			
Cell surface charge	PEG	E = M = H	[172]			
Density based centrifugation followed by invasion	САМ	M > E	[173]			
Negative selection		^				
Microfluidic size based then negative selection for CD45	CTC-iChip; Cytelligen® and iFISH	$\mathbf{E} = \mathbf{M} = \mathbf{H}$	[162, 163]			
Density separation of tetrameric antibody complexes for CD45, CD66b and glycophorin	RARE	E = M = H	[164]			
Density gradient separation then anti-CD45 based negative immunomagnetic enrichment	unnamed	E = M = H	[165]			
Positive selection						
Cell surface vimentin	CSV 84-1	М	[118]			
Cell surface EpCAM and FRα	unnamed	E = M > H	[174]			
High throughput microscopy for immunofluorescence or FISH	Epic CTC Platform®; FAST	E = M > H	[175–177]			
EpCAM based immunomagnetic separation	CellSearch®	E	[170]			
Microfluidic size based then EpCAM based immunomagnetic separation	CTC-chip; Herringbone; eDAR; OncoBean	Е	[178–181]			
Sized based filtration then EpCAM, CK, vimentin, and twist RNA-ISH	CanPatrol	E = M > H	[94]			
Flow Cytometry for surface epithelial markers	IE/FC	E	[182]			
Filtration using selective size amplification	SSA-MOA	E	[183]			
Clusters						
Size based filtration	FMSA; Cluster-Chip	M = H > E	[143, 144, 184]			

Table 2.2 Methods of CTC isolation and EMP recovery

2.3 Clinical Correlates and Future Study

2.3.1 EMP as a Biomarker for Progression, Aggressiveness, and Drug Selection

The functional connections between EMP and cancer progression are well-established and are supported by prognostic correlations observed in patient-derived samples. In ovarian cancer, higher EMP scores are correlated with worse prognosis, both for overall and disease-free survival [156]. In metastatic breast, pancreatic, and hepatocellular carcinomas, increases in EMP CTCs are associated with progression, poor therapeutic response, metastasis, and worse prognosis while patients responding to therapy show a decrease in EMP CTCs [7, 19, 43, 80, 93, 95, 127]. The hybrid-EMP phenotype predominates in many cancer types, including aggressive breast cancer and melanoma, and may therefore indicate a worse prognosis than tumors with a purely mesenchymal phenotype [80]. Further, cancer cells exhibiting hybrid-EMP were more plastic, and more efficient in tumor budding, invasion, stemness, CTC cluster formation, and drug resistance [34, 51]. Because CTCs are hematogenously circulating and represent many stages of metastasis, evaluation of EMP in CTCs may have clinical relevance as a biomarker [93]. However, these studies are still preliminary and the prognostic value of EMP CTCs in monitoring therapeutic resistance or progression has not been fully determined and no recommendation for clinical monitoring has been issued [127, 185].

2.3.2 Prevention or Reversal of EMP as a Therapeutic Target

In addition to serving as a biomarker, EMP may be an attractive therapeutic target to slow or halt metastasis. Current clinical trials aiming to prevent or reverse EMT are testing TGF β inhibition (LY2157299 in glioblastoma and hepatocellular carcinoma), clusterin (a TGF β mediator) inhibition (AB-16B5 in advanced solid tumors), platelet inhibition (aspirin in metastatic breast and colorectal cancer), AXL inhibition (TP-0903 in refractory solid tumors), and Src kinase inhibition, with mixed results [186–192]. Reversing transition to a mesenchymal state through redifferentiation could reduce invasiveness and resensitize cells to current therapies. However, there are concerns associated with therapies targeting EMP. First, MET is likely required for outgrowth at the secondary site and therefore such a treatment may actually support metastasis, possibly through reactivating dormant tumor cells [47, 83, 193-196]. Indeed, knockdown of the EMP transcription factors PRRX1 and Twist1 in breast cancer cells increased lung metastasis in mice [47]. Reciprocally, induction of Twist1 in a skin cancer model inhibited metastatic outgrowth [83]. Second, even if we could be confident that EMP inhibition would not be detrimental to the patient, the benchmarks for such a reversal are unclear. As described, EMP is not a single phenotype, but a broad array of intermediate states in different cells. It is therefore difficult to determine how far along the EMP continuum to reverse the cells, and how to achieve consistent effects in such a heterogeneous population. The best course of action will likely be different for different contexts and cancer types, further complicating the issue [7].

2.4 Controversies

Two papers published in 2015 using lineage tracing mouse models raised doubts about whether EMP is strictly necessary for metastasis in vivo (although they maintain support for a role in chemoresistance) [12, 13, 30, 197]. However, numerous papers in response to these findings have drawn on decades of research in support of a role for EMP in metastasis, pointing out that the complexity of this dynamic process – with interactions between multiple transcription factors, important intermediate and hard to detect phenotypes, and necessary plasticity between epithelial and mesenchymal states to complete the metastatic cycle – makes it very difficult to interpret the results of a single lineage tracing model [7, 14, 15, 80, 195]. Future models of greater nuance relying on multiple EMP markers and single cell analysis will help to fully understand the role of EMP in metastasis.

2.5 Remaining Questions

Despite over 30 years of study, new and old questions remain to be addressed to clarify the role that EMP, and therefore CTCs and CTC clusters, play in metastasis. As we continue to probe further into the impact of EMP on tumorigenesis and metastasis, our increased awareness of the hybrid-EMP phenotypes exhibited by many tumor cells, but especially CTCs, will provide more insight into this process. Further studies are needed to define how many distinct subtypes there are within the continuum, how stable/plastic these subtypes are relative to each other, and whether their functional characteristics remain the same across different cancer types. This will require a collaborative decision regarding the markers of epithelial and mesenchymal phenotypes, the setting of thresholds for expression, and establishing of assays that mimic interconversion between states in patients. It will also need to be determined whether these hybrid-EMP subtypes are best modeled as a continuum or as a trans-differentiation. This will be informed by studies examining how cells transition between the subtypes, including examinations of both transcriptional post-transcriptional and regulations.

Beyond defining hybrid-EMP, it is becoming clear that hybrid-EMP in CTCs and tumor cells alike is correlated with a worse prognosis and higher metastatic potential that fully epithelial or mesenchymal cells [53, 129]. It remains to be determined whether it is the hybrid-EMP cells themselves, or just the existence of a more heterogeneous population of tumor cells, that is the cause of this observation. On the one hand, metastasis requires both mesenchymal and epithelial processes, and cells locked into a mesenchymal state may fail to initiate a tumor in the secondary site. It is possible that hybrid-EMP CTCs encompass the population of CSCs that are the crucial determinants of successful tumor reinitiation. On the other hand, different cancer types and individual cancers exhibit different levels of hybrid-EMP, and yet many cancers are metastatic. With the recent identification of CTC clusters and the appreciation of their higher metastatic potential, it is possible that heterogeneous clusters of CTCs, containing cells with epithelial, hybrid-EMP, and mesenchymal phenotypes can form and cooperatively make the metastatic journey, with the mesenchymal cells "shepherding" the epithelial cells to their destination. Our ability to address these and other mechanistic questions will be aided by technological developments. Already, CTC isolation technologies have given us an opportunity to study some of these questions in the most appropriate setting – invaded cells that are the putative precursors of metastasis. Because these rare cells must be enriched, it will be crucial to select the appropriate isolation technology so that our evaluation of the breadth of EMP phenotypes in CTCs is not biased. To confidently accomplish this, we will need to standardize epithelial, mesenchymal, and CTC markers. Upon isolation of a physiologically relevant CTC population, advances in genomics and proteomics will allow for comprehensive mapping transcriptional, epigenetic, of and posttranscriptional differences in EMP phenotypes in individual CTCs and throughout disease progression. Finally, although CTCs are the metastatically competent population, upon isolation they still provide only a snapshot in the EMP progression of that cell. As with all EMP studies, animal models and lineage tracing technologies will be crucial to visualize and ultimately understand the implications of EMP on metastasis in vivo.

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3

Disseminated Tumor Cells and Dormancy in Breast Cancer Progression

Malgorzata Banys-Paluchowski, Florian Reinhardt, and Tanja Fehm

Abstract

Hematogenous dissemination of single cancer cells is a common phenomenon in patients with solid tumors. These cells may experience different fates: most will die during the process; some will grow into metastasis and some will persist in secondary homing sites for many years in a state referred to as dormancy. The mechanisms of this state are still not clear; single cancer cells can survive either by completely withdrawing from the cell cycle or by continuing to proliferate at a slow rate that is counterbalanced by cell death. Another hypothesis assumes that at least some of dormant tumor cells feature stem celllike characteristics that may contribute to their extremely long half-lives and enhance chemotherapy resistance. Breast cancer is particularly known for prolonged periods of clinical freedom of disease (sometimes up to 20-30 years), followed by a distant relapse. In this chapter, we explore the relationship between the clinical phenomenon of tumor dormancy and the disseminated tumor cells and discuss the potential implications for treatment.

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Keywords

Breast cancer · Disseminated tumor cells (DTCs) · Circulating tumor cells (CTCs) · Cancer dormancy · Angiogenesis · Microenvironment · Metastasis

3.1 Introduction

Breast cancer (BC) is the most commonly diagnosed cancer and the second leading cause of death due to malignant disease in women worldwide. Despite adequate surgical and (neo) adjuvant systemic treatment, approximately one out of three to one out of four patients develops a relapse over time [1], suggesting that single tumor cells or tumor cell clusters, sometimes referred to as minimal residual disease (MRD), may survive at secondary sites and lead to tumor growth several years later [2]. The theory of hematogenous spread of solid tumors has been introduced by several researchers as early as nineteenth century, based on autopsy studies and the detection of cancer cells similar to those from the primary tumor in the blood [3, 4]. In the late twentieth century, the MRD research focused mostly on tumor cells found in the bone marrow. These disseminated tumor cells (DTCs) can be routinely detected in up to 40% of patients with primary BC and their presence predicts shorter

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disease-free and overall survival [5]. Further, a subset of these DTCs have been shown to survive chemotherapy; their persistence is associated with impaired clinical outcome as well [6, 7].

The development of improved assays for single cell detection and the introduction of new enrichment methods have enabled the research focus to shift to peripheral blood as an alternative compartment hosting tumor cells. The major advantage over bone marrow is the easy accessibility without the necessity of an invasive procedure and the possibility of serial measurements. When encountered in the blood, cancer cells are usually referred to as circulating tumor cells (CTCs). Currently, the overwhelming majority of studies registered in the ClinicalTrials.gov and EudraCT registries focuses on the CTCs in the blood; only six studies concern DTCs in the bone marrow [8]. With regard to their clinical relevance, CTCs have been shown to predict worse survival in both metastatic and early BC in large meta-analyses [9, 10].

In this chapter, we will discuss the role of DTCs in cancer dormancy and the clinical implications of this phenomenon.

3.2 Cancer Dormancy

Tumor dormancy, a phenomenon well-known to clinical oncologists, refers to a period of time in which tumor cells are assumed to be present but disease progression is not clinically apparent. BC is one of the entities known for prolonged asymptomatic periods, sometimes as long as 20–25 years, followed by a recurrence [11, 12]. About 20% of clinically disease-free breast cancer patients suffer from a relapse 7-25 years after mastectomy. Between 10 and 20 years after surgery, the rate of recurrence is relatively steady at about 1% per year [11, 13]. Similar courses of disease have also been observed in melanoma, prostate, thyroid and renal carcinoma, while late relapses are comparatively rare in colon and lung carcinoma [14]. As dormant single cells or micrometastases at secondary homing sites are widely assumed to be precursors of disease progression, their detection and possible elimination with adjuvant targeted therapies is a major goal of care of BC survivors.

Late recurrences might be due to the ability of DTCs to survive in a dormant state, evade therapies and finally transition to a proliferative state. Indeed, Meng et al. were able to detect single tumor cells in the blood in 36% of asymptomatic and clinically disease-free BC patients 7-22 years after diagnosis [15]. Recently, two large trials prospectively investigated the clinical relevance of CTC persistence. Sparano et al. showed that 4.8% of patients with non-metastatic BC had at least one CTC/7.5 ml blood around 5 years after diagnosis; these patients had a risk of relapse that was 18 times higher than that of CTC-negative women [16, 17]. Interestingly, CTC status was the strongest predictor of disease recurrence in the multivariate analysis. Similar results were reported in the German SUCCESS A trial [18]. In patients with hormone receptor positive BC, the CTC status 5 years after diagnosis significantly predicted shorter disease-free survival.

Yet, despite DTCs/CTCs being an independent prognostic predictor, the majority of patients with minimal residual disease does not develop metastases [5]. One possible explanation might be the phenomenon called "metastatic inefficiency". Although large numbers of cancer cells enter blood circulation every day [19, 20], most are already apoptotic or dead and it is currently assumed that less than one cell out of a thousand might give rise to subsequent secondary growth [21, 22]. Possibly, a significant proportion of viable tumor cells might be eliminated after entering blood vessels by shear mechanical forces of the blood stream [19, 23, 24].

There are currently no markers available to exactly predict the risk for late recurrence. Furthermore, it is not possible to predict which dormant tumor cells or micrometastases will eventually grow and which will stay dormant without ever becoming clinically relevant.

3.3 Potential Mechanisms and Clinical Relevance of Tumor Cell Dormancy

Despite major advances in therapy of BC leading to improvements in relapse-free and overall survival, a population of tumor cells is able to survive systemic chemotherapy or targeted therapies and persist in blood or secondary sites. Cytotoxic treatment regimens especially target highly proliferative cells. In contrast, dormant tumor cells are mostly either slowly proliferating or remain in a state of quiescence, which is determined by the lack of proliferating markers (Ki-67, PCNA) accompanied by the lack of apoptotic markers (TUNEL, M30) and may explain the failure of conventional chemotherapy in some BC patients [25]. DTC dormancy was recently supported by DTCs expressing markers including NR2F1, DEC2 and p27 [26]. Of these, NR2F1 (Nuclear Receptor Subfamily 2 Group F Member 1) has been shown to play a key role in dormancy signaling [27]. DEC2 (also known as SHARP1 or BHLHE41) is a metastasis suppressor and is assumed to induce dormancy by activating p27 [28]. Moreover, current findings indicate that a subset of DTCs in breast cancer patients undergoes an epithelial-to-mesenchymal transition (EMT) and obtain a stem cell-like phenotype. DTCs that hold a stem cell-like phenotype (e.g. expression of ALDH, presence of CD44 and absence of CD24) are called cancer stem cells (CSCs) [29, 30]. A stem cell-like phenotype might be responsible for their resistance to cytostatic therapy [6, 31]. New treatment strategies that emerge from understanding the biology of dormant tumor cells include the ability to induce or maintain dormancy and induce the programmed cell death. Based on current dormancy studies, potential therapeutic strategies include: altering the microenvironment, targeting angiogenesis, targeting signal transduction and activating the immune system.

3.3.1 Microenvironment

Several clinical and pre-clinical studies have provided ample evidence that not only the cancer itself but also the tumor microenvironment plays a significant role in BC progression, metastasis and therapeutic outcome. Cancer cells are surrounded by various other cells with which they stay in constant interaction. The tumor microenvironment (TME) comprises of cancer cells, cancer associated fibroblasts (CAFs), endothelial cells and pericytes, immune and inflammatory cells, bone marrow derived cells and the extracellular matrix [32, 33]. The bidirectional cross-talk between cancer cells and the TME determines the extent of cell proliferation, angiogenesis, invasion and survival. Systemic treatment should therefore not only target cancer cells but also the surrounding TME. Treatment options are bisphosphonates (BPs) or the RANKL inhibitor denosumab, which are potent inhibitors of osteoclast-mediated bone resorption. Beyond their traditional use in bone metastatic disease, in vitro as well in vivo studies support a possible role as anticancer therapies by preventing cancer cell migration, and by promoting cancer cell death by changing the bone into a "hostile" environment. BPs and denosumab influence the TME by altered secretion of growth factors as well as cytokines and may act indirectly on cancer cells through microenvironmental changes using immunomodulatory and antiangiogenetic effects. Several studies confirmed the efficacy of BPs in preventing new bony and visceral metastases and their positive impact on progression-free and overall survival in selected BC subgroups (ABCSG-12, patient ZO-FAST, AZURE, NSABP B-34 trial) [34-38]. Small pilot studies have already demonstrated that BPs contributed to eliminate dormant DTCs, even after years of first diagnosis [39-42]. Moreover, the DTC status might be predictive of the efficacy of bisphosphonate therapy [43]. A current nonrandomized phase II pilot study is evaluating the impact of denosumab on DTCs in patients with primary BC (NCT01545648). Patients with persistent DTCs received denosumab monthly for 6 months, then every 3 months for a total of 1-year treatment. To date, there are no published results yet.

While hypoxia is a poor-prognosis microenvironmental feature of solid tumors, it also seems to play an important role in tumor cell dormancy. One of the early responses to oxygen deficit is the reduction of oxygen consumption, achieved by decreased proliferation allowing cells to stay viable for long periods of time while dividing very slowly [44]. Primary tumors exposed to hypoxic microenvironments have been shown to upregulate both hypoxia and dormancy genes. Interestingly, once cancer cells left the primary tumor, the expression of dormancy markers persisted, but the hypoxic response did not, suggesting that the dormancy-like response lasts longer than the hypoxic program [26]. Cell line-based studies have also demonstrated that repeated hypoxia leads to development of breast cancer cells adapted to hypoxic state by entering a dormant state [45].

3.3.2 Angiogenesis

Angiogenic dormancy can be defined as the state in which tumor cell proliferation is counterbalanced by apoptosis owing to poor vascularization. The lack of tumor angiogenesis impedes tumor growth beyond a microscopic size (2-3mm), resulting in an asymptomatic and nonmetastatic state [46]. The angiogenic switch of cancer cells from a dormant, non-angiogenic phenotype to an active, angiogenic phenotype is a critical step and essential to promote fastgrowing and expansion of tumor masses. Angiogenesis is therefore a critical feature of tumor growth and inhibition a potential treatment method. There are many growth factors involved in the physiological regulation of blood vessel formation. Blockade of even a single growth factor might limit vascular growth, with the most compelling evidence to date supporting blockade of VEGF. Several clinical trials on bevacizumab, a monoclonal antibody against VEGF, have shown improved progression-free survival when administered in combination with chemotherapy in the metastatic setting (E2100, RIBBON-1, AVADO) [47–49]. However, the overall survival was not affected. In early breast cancer, clinical studies on bevacizumab did not demonstrate a disease-free or overall survival benefit (ARTemis, GeparQuinto trial) [50, 51]. Besides bevacizumab, small inhibitors of VEGFR receptor tyrosine kinases (sunitinib) either alone or in combination with chemotherapy showed no clinical benefit for patients with advanced breast cancer [52]. Future trials might

help to clarify whether prevention of the angiogenic switch with antiangiogenic agents might achieve clinically relevant results in terms of elimination of dormant tumor cells.

3.3.3 Targeting Signaling Pathways

Once dormant tumor cells leave their quiescent state, they may express specific receptors which, when activated can initiate downstream signaling resulting in the expression of genes for cancer cell proliferation, growth, survival, migration, and other vital cell cycle pathways. There is an increasing amount of targeted therapies which interfere with the function of specific molecules responsible for tumorigenesis and cell cycle.

The human epidermal growth factor receptor 2 (HER2) is one of the main targets. Several studies revealed that HER2 expression on both DTCs and CTCs differed from HER2 expression of the primary tumor and HER2 expression on DTCs and CTCs was correlated with poor prognosis [53–60]. During disease progression, HER2 gene amplification can be acquired even if the primary tumor was negative for HER2. Based on these observations, two pilot studies showed that adjuvant trastuzumab treatment is able to eliminate DTCs and CTCs [61, 62]. Yet, the recently published randomized TREAT CTC trial and the NSABP-B47 trial both failed to confirm the hypothesis that adjuvant trastuzumab can benefit women with HER2 non-amplified early breast cancer [63, 64].

The expression of the estrogen receptor (ER) on cancer cells is another main factor because endocrine adjuvant therapy remains a cornerstone of breast cancer treatment. In line to HER2, several studies have revealed a discordance of ER status between primary tumor and DTCs as well as CTCs [60, 65, 66]. This might be relevant for clinicians when selecting patients for adjuvant endocrine therapy. A loss of ER-positivity of MRD might explain the failure of adjuvant endocrine therapies in a subgroup of ER-positive BC patients. Moreover, the discordance could be important for patients lacking ER on the primary tumor but showing ER-positive DTCs/CTCs because they might benefit from an endocrine therapy. Determining the phenotype of DTCs and CTCs is therefore becoming more and more important, as occult tumor cells are the targets of all adjuvant treatment regimes. Besides local treatment of the primary tumor and lymph node metastases, the definitive success of BC therapy is dependent on the ability to eliminate residual cancer cells which are persistent after primary surgery, before they become clinically evident.

There are increasing numbers of other specific agents targeting the signal transduction, including everolimus (mTOR inhibitor), lapatinib (EGFR and HER2 inhibitor), pyrotinib (HER1, HER2, and HER4 inhibitor), pertuzumab (HER2 dimerization inhibitor), ribociclib/abemaciclib/palbociclib (cyclin-dependent kinase 4/6 inhibitors), T-DM1 (combination of trastuzumab and the chemotherapy medicine emtansine) and alpelisib (an α -specific PI3K inhibitor). The ability to determine and monitor the biology of MRD cells and to follow changes on proteomic, transcriptomic and genomic level in real-time may allow the tailoring of conventional medical treatment to individual characteristics. However, clinical studies demonstrated that elimination of dormant tumor cells may not directly impact the survival. Prospective randomized controlled trials are therefore needed to investigate whether patients with persistent MRD benefit from these agents.

3.3.4 Immune System

The inherent capacity of the immune system has a major impact on the balance between dormant tumor cells and tumor growth. The dynamic process consisting of immunosurveillance and tumor progression, referred to as *immunoediting, is made up of three phases*: elimination, equilibrium, and escape [67]. In the equilibrium phase, the immune system holds tumor cells in a state of functional dormancy or quiescence by hostderived cytotoxic T lymphocytes [68]. Various approaches have been developed to sustain such endogenous host-protective immune responses

including immunomodulating antibodies which specifically block immune checkpoint inhibitors and potentially expand endogenous anticancer immune responses. Most promising immunomodulating antibodies are monoclonal anti-PD-1 (pembrolizumab) or anti-PD-L1 (atezolizumab, durvalumab) antibodies for the treatment of patients with advanced triple negative breast cancer. Clinical trials showed objective response rates in the 5%-19% range [69-71]. Hostprotective immune responses can be also amplified by vaccines, which boost naturally occurring antitumor immune responses. Many different types of cancer vaccines have been constructed from distinct immunogenic sources represented by whole tumor lysates, tumor antigenic peptides, DNA, RNA, and viruses. Moreover, they can be combined with immunoadjuvants, which contribute to the immune stimulation. Encouraging results are coming out during several clinical phase II/III trials. NeuVax, AVX901, and INO-1400 are currently the most promising BC vaccines [72]. In (dormant) MRD, favorable effector-target ratios prevail and therefore might be optimally suited for vaccines and immunotherapy with antibodies.

3.4 Conclusions

Tumor dormancy is a clinically relevant phenomenon that reflects the ability of minimal residual disease to elude systemic therapy and persist as single cancer cells or micrometastasis at secondary homing sites. Dormant cells can either completely withdraw from cell cycle and remain in mitotic arrest or divide at a very slow rate counterbalanced by cell death. However, the exact mechanisms underlying tumor dormancy and leading to activation of dormant cells are still unclear. Possibly, angiogenetic and immunomodulatory factors contribute to the development of a microenvironment most suitable for hosting dormant cells. To effectively target these cells, better understanding of tumor dormancy is necessary and might help to design new targeted approaches to control this step of disease progression.

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GENISTA

Methodology for the Isolation and Analysis of CTCs

Clotilde Costa and Ana B. Dávila-Ibáñez

Abstract

The majority of deaths related to breast cancer are caused by metastasis. Understanding the process of metastasis is key to achieve a reduction on breast cancer mortality. Currently, liquid biopsies are gaining attention in this regard. Circulating tumor cells (CTCs), an important component of liquid biopsies, are cells shed from primary tumor that disseminate to blood circulation being responsible of distal metastasis. Hence, the study CTCs is a promising alternative to monitor the progress of metastasis disease and can be used for early diagnosis of cancers as well as for earlier assessment of cancer recurrence and therapy efficacy. Despite their clinical interest, CTC analysis is not recommended by oncology guidelines so far. The main reason is that there is no gold standard

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A. B. Dávila-Ibáñez (⊠) Roche-Chus Joint Unit, Translational Medical Oncology Group (Oncomet), Health Research Institute of Santiago de Compostela, Santiago de Compostela, Spain e-mail: ana.belen.davila.ibanez@sergas.es technology for CTCs isolation and most of the current technologies are not yet validated for clinical use. In this chapter we will focus on the most relevant technologies for CTC isolation based on their properties and depending on whether it is a positive or negative selection. We also describe each technology based on its potential use and its relevance in breast cancer. The chapter also contains a future perspective including the challenges and requirements of CTC detection.

Keywords

Breast cancer · Circulating tumor cells (CTCs) · CTC capture · CTC enrichment · Detection · Technology

4.1 Introduction

The vast majority of deaths related to breast cancer are caused by distant organ metastasis. The metastasis of this tumor type is mainly established by the hematogenous dissemination of tumor cells from the primary tumor. Thus, understanding the process of metastasis is key to achieve a reduction on breast cancer mortality.

The current methods used in clinical practice to monitor the disease mainly involve tissue biopsy, imaging techniques and evaluation of



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serum markers such as CA 15-3 and carcinoembryonic antigen (CEA). However tissue biopsy is invasive for patients since entails surgery, the material obtained usually is limited, and does not represent the intratumoral heterogeneity while imaging techniques show limited sensibility. These approaches have been shown to have a limited success, however, in recent years the technic known as "Liquid biopsy" and within this, the study of the circulating tumor cells (CTCs), has emerged as an useful alternative for disease monitoring. CTCs can be isolated from the blood of patients in a longitudinal and non-invasive manner, providing real time information about the status of the disease. In this regard, the prognostic value of the enumeration of CTCs in patients with metastatic breast cancer (MBC) and even in early breast cancer, has been extensively proved [1–3]. Despite of these evidences, CTC enumeration is not currently recommended by oncology guidelines, meaning that further clinical validation is still needed for this approach. However, efforts are being made to advance on the development of technologies used for CTC isolation, in the quest for higher sensitivity and specificity.

The possibility of isolating CTCs for enumeration and analysis is conditioned by the currently available technologies. Although in recent years there have been great advances in this regard, there are still important challenges to be considered. CTCs have an estimated blood frequency ranging from 1 to 10 CTC in 10⁶-10⁸ blood cells depending on the tumor stage. CTCs are "rare" events in blood, so there is a need for a high sensitive and specific technology for their detection. Another important limitation to take into account is the sample volume to be processed in order to guarantee a successful isolation of CTCs. Additionally, CTCs can be shed either by the primary tumor or metastatic sites, thus showing high heterogeneity regarding expression of markers (epithelial or mesenchymal), as well as different cell sizes, morphologies and plasticity. All this together makes difficult to isolate pure CTCs in one-step approach. To achieve the implementation of CTCs in routine clinical practice, since CTCs enumeration alone is not enough, it is needed to get relevant clinical information out of them, hence improving patient outcome. Ideally, the selected technology for CTCs isolation should also be suitable for downstream analysis such as molecular characterization or functional assays. In fact, the main advantage of CTCs analysis over other circulating biomarkers such as ctDNA is the possibility to perform RNA expression studies, known as transcriptomics, as well as other "omics" analyses.

In this chapter we will review the most relevant technologies used for the isolation of CTCs from the blood of cancer patients, with particular emphasis on those applied for the study of breast cancer dissemination.

4.2 Isolation of CTCs: Enrichment

An ideal CTC detection platform must be capable of isolating and detecting the different subpopulations of CTCs, discarding the background contamination of blood cells. The first step is usually the enrichment of CTCs, which allows the separation of CTCs from blood cells, followed by a step of distinction or identification (and possibly characterization) of the CTCs that allows to determine the tumoral nature of the cells. Currently, there is no system capable of isolating a pure population of CTCs, and indeed, most available technologies concomitantly isolate in a non-specific manner some cells from the hematopoietic fraction together with CTCs. Thus, depending on the purity of the sample obtained, the approach is considered of high or low specificity. To select the most accurate isolation technique it must be taken into account the specificity and sensitivity, as well as the desired downstream analysis to be performed (enumeration, molecular characterization or the performance of functional assays). In addition, the origin of the sample to analyse must also be considered, whether it comes from blood, urine or another biological fluid, and its collection procedure and storage (e.g. if includes a fixation step or not).

The phenotypic characterization of the cells is usually performed using immunofluorescence techniques that permit the simultaneous visualization of different markers. Thereby, cells are immunostained for epithelial, mesenchymal or tumor specific, and blood cell markers, as well as nuclear staining which permits to check the integrity of the cell. The immune-labelling of cells provides additional information about morphology, size and fitness, for example whether they have entered apoptosis (vesicular cells), or about the phenotype (epithelial or mesenchymal traits) [4]. Additional downstream options for molecular characterization of the cells are Copy Number Variation (CNV) analyses or Next-Generation Sequencing (NGS) to verify the tumor nature of the cells. For a more in depth characterization, single cell level analysis is the most suitable methodology to avoid biased results due to low specificity associated to general approaches isolating pools of CTCs.

Current methods for CTC isolation can be divided in two main blocks according to the strategy used for the capture of CTCs, based on (1) biological and (2) biophysical properties of the cells (Table 4.1).

4.2.1 Strategies Based on Biological Properties

The most common technique is the immunoisolation which is based on the use of antibodies against cell surface markers. It can be directly applied to the whole blood or to a previously isolated fraction of peripheral blood mononuclear cells (PBMCs), usually obtained by a density gradient centrifugation protocol. CTCs can be isolated through positive selection, targeting tumor-associated antigens expressed by CTCs; or negative selection, removing background cells by targeting antigens expressed by them but not by CTCs.

4.2.1.1 Positive Selection

Positive selection, the target molecule is an epithelial surface antigen, such as EpCAM (epithelial cell adhesion molecule), HER2 (human epidermal growth factor receptor 2), EGFR (endothelial growth factor receptor) or CEA. Likewise, other mesenchymal markers such as cell surface vimentin [5, 6] and N-cadherin [7], and stem cell marker CD113 have been used to isolate non-epithelial cell populations given the importance of the epithelial-to-mesenchymal transition (EMT) and stem cell markers in different subpopulations of CTCs in relation to metastasis. For the isolation of CTCs from breast cancer patients, the most commonly used antigen for a positive selection is EpCAM, which is a surface protein expressed in epithelial cells. It is assumed that under physiological conditions epithelial cells do not circulate in the bloodstream, thus the presence of EpCAM-positive cells would have to come from tumor cells released into the circulation.

CellSearch® system (Menarini Silicon Biosystems), is one of the most commonly used systems based on immunoisolation, and it is the only system approved by the Food and Drug Administration (FDA) for the isolation and detection of CTCs in metastatic breast, prostate and colorectal cancer. This system uses whole blood and bases the isolation of CTCs on the expression of EpCAM, using particles with a magnetic core functionalized with anti-EpCAM antibodies. EpCAM-positive cells are then sorted in a magnetic field and immunostained for cytokeratins (CKs) and CD45. The current CellSearch® system defines a CTC as an event that has a nucleus (DAPI positive); expresses cytokeratins (CK8, CK18 and CK19); does not express CD45 and it is more than $4 \times 4 \ \mu m^2$ in size. This system is mainly used for CTC enumeration, and since blood samples are usually collected in tubes containing fixatives for cell preservation, cells isolated by CellSearch® would no longer be suitable for downstream gene expression or functional analysis. However enriched samples could be used for additional phenotypic characterization or subsequently single cell isolation for DNA sequencing, in combination with other specific technologies.

Although CellSearch® system is widely used for CTCs enumeration, it presents some drawbacks since it only detects CTCs in about 70% of MBC patients [8], and in the non-metastatic setting the sensitivity is much lower. This limitation could be partially explained by the loss or low EpCAM expression in some tumor cell populations (mesenchymal or stem subpopulation).

POSITIVE SELECTION	Methodology	Advantages	Disadvantages	
	CellSearch®	FDA approved Analysis can be made from whole blood Allows fluorescence analysis Clinical relevance, semiautomatic	Based on the expression of cell surface proteins(EpCAM) Expensive equipment Viable cell recovery is not possible	
	AdnaTest	Allows gene expression analysis High sensibility	Based on the expression of cell surface proteins (EpCAM, CA 15-3 (MUC1), HER2, ER and PR (optional))	
	Dynabeads	Allows the isolation by custom antibodies Isolate viable cells	Based on the expression of cell surface proteins (EpCAM and others)	
	CellCollector®	CE approval In vivo sample processing allows the screening of high blood concentrations Isolate viable cells	Based on the expression of cell surface proteins (EpCAM) More invasive for the patients, nowadays is not implemented due the limitations of the time needed for the analysis	
NEGATIVE SELECTION	RosetteSep™	Isolate viable CTCs Independent of epitope expression	Antibody-labelling alters cell density	
PHYSICAL PROPERTIN	ES			
DENSITY	Methodology RareCyte® LeukApheresis, Ficoll-Paque™	Advantages Independent of equipment, faster Independent of	Low specificity and efficiency (CTCs loss in plasma fraction)	
RE		surface proteins Isolate viable cells LeukApheresis allows the screening of high volumes of blood	Higher "contamination" with leukocytes	
FILTRATION	ISET®, MetaCell®, ScreenCell®, Celsee Genesis system	Preserves integrity Shorter times of sample processing Independent of surface proteins	Processing of the sample need to be done within 4 hours of collection Possible blood clogging on the filter which stops filtration.	
MICROFLUIDICS Label-free & label-based Label-free & label-based EX1, CTC-Chip, LiquidBiopsy®, Target Selector TM , IsoFlux, ^{HB} CTC-Chip, CytoTrapNano TM		Isolate viable cells Controllable and tunable flow patterns Offer ability to multiplex platforms Easy operation	Clogging problems can limit the flow	
DIELECTROPHORESIS	ApoStream®, DEPArray™	Viable cell isolated Independent of surface proteins	High-intensity electric field - step-by-step operation needed	

 Table 4.1
 Technologies for the isolation of CTCs

(continued)

DEPArray [™] , VyCAP, Celsee	Single CTCs and	Requires high sample
Genesis system	cluster detection	processing time (except
	Allows single cell	VyCAP)
	molecular	
	heterogeneity analysis	
	DEPArray™, VyCAP, Celsee Genesis system	DEPArray TM , VyCAP, Celsee Single CTCs and Genesis system cluster detection Allows single cell molecular heterogeneity analysis

Table 4.1 (continued)

FDA Food and Drug Administration, *CE* abbreviation of French phrase "Conformité Européene" which literally means "European Conformity", *EpCAM* epithelial cell adhesion molecule, *HER2* human epidermal growth factor receptor 2, *ER* estrogen receptor, *PR* progesterone receptor

Regarding its performance in the different molecular breast cancer subtypes, it has been described that CTCs are mainly detected in Luminal subtype and rarely in Triple Negative (TN) patients. Despite of these limitations, the presence of ≥ 5 CTCs/7.5 ml blood was shown to be significantly associated with a shorter overall and progressionfree survival (OS, PFS) in MBC patients [9]. Some studies had supplied evidence that the positivity of CTCs 5 years after the diagnosis of Luminal BC provided independent prognostic information for late clinical recurrence [10]. In addition, the prognostic relevance of the CTCs before and after adjuvant chemotherapy has been demonstrated (Success Study Group) [11] and even 2 years after chemotherapy or neoadjuvant therapy [12]. Thus, the prognostic value of CTCs detected by CellSearch® technology is clear in BC patients, which have encouraged other technologies to improve the efficiency of detection of CTCs in MBC patients and to offer other alternatives for downstream analyses.

AdnaTest (Adnagen, Qiagen), is an immunebased method for CTCs isolation and gene expression analysis. It consists of a kit containing magnetic beads conjugated with a cocktail of antibodies (EpCAM, CA 15-3 (MUC1) and HER2; and optional detection of ER and PR), specifically optimized for BC. This system is used for gene expression analysis by real-time multiplexed PCR (RT-PCR) of a panel of relevant tumor markers and characteristic of this tumor type. This technology has been used to identify gene expression signatures in CTCs from MBC patients in association to therapy response [13, 14], and interestingly it has shown that ERBB2 overexpression in CTCs from patients with HER2-negative primary tumors significantly associates with disease progression [14].

CellCollector® (Gilupi) and Dynabeads (CELLectionTM Epithelial Enrich DynabeadsTM; ThermoFisher) are two other methodologies for immune isolation of CTCs based on EpCAM recognition. CellCollector® is the first in vivo CTC-isolation product that has CE approval. It is a medical wire coated with anti-EpCAM antibodies directly placed in the bloodstream of a patient through a permanent catheter (size 20 G). It remains inserted in the vein of the arm for 30 min, getting in contact with a larger volume of blood and allowing the capture of CTCs in vivo. In a study with BC patients, this system successfully enriched EpCAM-positive CTCs in 83.3% of patients, with a median of 5.5 (0-50) CTCs. CTCs were not detected in healthy volunteers but could be isolated from patients at early stages in whom distant metastases have not yet been diagnosed [15]. However, its implementation in the clinic is not simple as it requires manual screening for the detection of CTCs. Dynabeads are EpCAM-coated magnetic beads added to the blood sample and allowed to interact for a short time, then the cells bound to the beads (CTCs) are separated with a magnet. Dynabeads have allowed the detection of EpCAM-negative/low CTCs from MBC patients by customizing the beads coating with different antibodies specific for surface proteins and extracellular matrix proteins [16]. Moreover, this technology has been used to detect CTCs with epithelial-mesenchymal transition and stemness features from BC patients [17].

Both systems, CellCollector® and Dynabeads, allow the recovery of viable cells which are suitable for subsequent characterization through assays including gene expression analysis and cell culture.

4.2.1.2 Negative Selection

Negative selection, it is based on the depletion of hematopoietic cells by targeting specific antigens of this cell lineage, allowing the enrichment of circulating epithelial cells. The most commonly used antigen is CD45 that is expressed in hematopoietic cells. Under these strategies, the nontargeted fraction of cells is the one of interest.

RosetteSepTM (EasySepTM Direct Human CTC Enrichment Kit, Stemcells), one of the most commonly used methodologies for negative immunoisolation [18]. This is an immunodensity cell isolation kit designed to enrich circulating epithelial tumor cells from fresh whole blood. The kit contains a cocktail of antibodies that recognizes blood cell antigens. There is a specific version of RosetteSepTM for breast cancer samples that includes the anti-CD56 antibody. Upon a density gradient centrifugation, the unwanted cells settle, and purified tumor cells are present as a highly enriched population at the interface between the plasma and the density gradient medium. Cells obtained are suitable for downstream analyses such as molecular phenotyping, gene expression or single cell analysis. In addition, as the recovered cells can remain viable, it is an optimal strategy for functional analysis, as demonstrated by Ramirez et al. who performed a subsequent secretome analysis using EPISPOT system [19], or by Baccelli et al. and Pereira-Veiga et al. who were able to generate CTCderived xenograft (CDX) mouse models from CTCs isolated from BC patients [20, 21].

4.2.2 Strategies Based on Physical Properties

The most important advantage associated to the technologies based on the physical properties of CTCs relies in the fact that they are independent of the recognition of surface markers ("label-free methods" or "epitope-independent methods"), therefore aimed to capture a more heterogeneous

population of cells. Contrary to immunoisolationbased techniques, this approach allows the isolation of cells with epithelial and mesenchymal phenotypes. Therefore these technologies are appropriate to isolate CTCs with EpCAM-low/ negative expression levels, as it occurs on triple negative (TN) BC patients. Additional, these technologies are less aggressive since they are not based in chemical interactions, thereby increasing cell viability. Technologies based on physical properties work by trapping the CTCs in a device to obtain an enriched population, while blood cells are discarded. However, there are some disadvantages related to this isolation approach, as it can cause the deformation and damage of CTCs by filter pores, as well as, the loss of those CTCs with smaller size than average. Also, CTCs have higher plasticity than normal cells so that they can squeeze and pass through the devices being undetected. In the other hand, larger size cells that are not cancer cells could be retained together with the isolated population, often contaminated with megakaryocytes, which are cells commonly found in the circulation of cancer patients who underwent chemotherapy [22, 23].

4.2.2.1 Density Centrifugation

Cell density is one of the first cell physical properties applied for the isolation of CTCs. Technologies based on this physical property take advantage of the differences in density between cell populations for the separation when submitted to a gradient centrifugation. Under these conditions, cells are retained in the buoyant per their relative density. However this methodology has several limitations being the most important the lack of specificity, which means the loss of some CTCs during the process. Currently, density gradient centrifugation is employed as a preliminary step prior to the application of another detection and isolation methodologies.

RareCyte® (RareCyte, Inc.) platform, integrates a density-based cell separation device (AccuCyte®) that allows the separation of the CTC-containing blood fraction due its density difference. This technology allows sample deposition onto microscope slides, automated multiparameter fluorescence staining, image scanning, analysis, review and mechanical CTC retrieval [24]. The platform utilizes six fluorescence channels, of which four of them are used to identify CTCs and two are available for custom markers. Single-cell retrieval from fixed slides is compatible with whole genome amplification methods for genomic analysis. This technology has been successfully used to collect CTCs from the blood of a breast cancer patient-derived xenograft (PDX) model, allowing for subsequent analyses which suggested a potential key role for Six1 (an EMT-inducing transcription factor) in metastatic dissemination [25].

Diagnostic LeukApheresis (DLA). The use of CTCs in the clinical practice remains a challenge due to their low frequency, particularly in the non-metastatic setting. DLA has been recently introduced as a more sophisticated strategy for the detection of CTCs. Leukapheresis is a standard clinical method based in the screening of liters of blood for the specific collection of cellular components with various applications such as stem cell harvest. DLA is presented as a possible solution to overcome the low frequency of CTC, since it allows the screening of large volumes of blood [26]. The basic principle of DLA is the collection of mononuclear cells (MNC) from peripheral blood by continuous centrifugation. Since epithelial cells have a similar density compared to MNCs [27], CTCs can be isolated together with the MNCs collected during this procedure. In a study comparing the prevalence of CTCs in DLA products (2 mL) with matched peripheral blood samples (7.5 mL) from different cancer types using the CellSearch® system, CTC could be detected in 72% of DLA samples as compared to a 28% in peripheral blood samples, and with a much higher CTC count per mL in DLA samples [28]. Moreover, analysis of DLA products from nonmetastatic BC patients showed that CTCs could be detected in 90% of the samples, and CTC frequency correlated to tumor stage. Importantly, CTCs present in DLA products are viable and after an enrichment step can be used to establish CTC cultures [29].

4.2.2.2 Filtration

Filtration is a size based methodology, wherein the blood cells can pass through the filtration device because are smaller than the pore size (6.5-8 μ m), while larger cells like CTCs are trapped. These technologies have the advantage that they work as a kit completely independent of any equipment. Some examples of commercially available technologies that isolate CTCs by filtration are:

ISET® (Isolation by SizE of Tumour cells; Rarecells diagnostics). This technology allows the isolation of rare cells and CTCs by blood vertical filtration of fixed samples, preserving cell integrity for further analysis. ISET® technology enables the isolation of CTCs and circulating tumor microemboli (CTM, potentially important cancer biomarkers; also referred to as CTC clusters) for almost all types of cancer (breast, lung, prostate, liver, etc.). Numerous clinical studies have chosen ISET® technology for isolation of CTCs [30]. A study published by Farace et al. proved clear discrepancies between CellSearch® and ISET® technologies with regard to the enumeration of CTCs in metastatic patients, including BC patients. The study showed that tumor cells undergoing EMT (characterized by the loss of epithelial markers and neoexpression of cytoplasmic mesenchymal markers) are not detectable by CellSearch®, whereas ISET® system is much more efficient at identifying these cells. Hence, the study has validated ISET® effectiveness for CTC isolation and proved how technologies based on the isolation of EpCAM-positive cells populations, show limitations, especially in patients with metastatic lung carcinoma [31].

MetaCell® (MetaCell s.r.o.), this size-based technology allows the filtration of up to 50 mL of blood through a membrane with pores of 8 μ m diameter. The technology is fast and the collection of the CTCs on the membrane takes 2 minutes for a 10 ml blood sample. It is a non-aggressive technology, thus, after the separation process, viable intact cells are suitable for subsequent characterization and/or in vitro cultivation over the filtration membrane. A study published by Jakabova et al. demonstrated the efficacy of

MetaCell[®] for the isolation of heterogeneous CTCs from BC patients, which have lost epithelial antigens as the result of the EMT process. In a cohort of 167 BC patients (stage I to III) they were able to detect CTCs in 76% of patients [32].

ScreenCell® (ScreenCell), is a technology for isolating circulating rare cells (including CTCs) from whole blood or other biological fluids that takes only 3 minutes. The technology is available in three different kits (ScreenCellCyto®, ScreenCell MB®, ScreenCell CC®). This system allows the recovery of live cells for further cytological studies such as enumeration and cell structure characterization, genomic analyses, and cell culturing for functional characterization. This technology has been shown to be useful for enrichment and identification of circulating tumor associated cells as well as for downstream genetic characterization of CTCs isolated from MBC patient [33].

4.2.2.3 Microfluidics

Nowadays microfluidics are one of the most popular technologies because they provide many attractive advantages for CTC studies such as continuous sample processing to reduce target cell loss, and easy integration of various functions as "do everything -on-a-chip". The isolation process is founded on the differences in size and deformability between CTCs and blood cells and the hydrodynamic flow applied in the devices.

Parsortix[™] (Angle plc), it is a low cost system and easy to manipulate, based in microfluidic technology, presented as a disposable cassette to capture and then harvest CTCs from whole blood. Cassettes have a critical gap size of 6.5 µm in which CTCs get retained. CTC capture is based on their larger size and less deformable nature when compared to other blood cell components. Additionally, it allows easy harvesting of CTCs providing viable cells for later staining and/or genetic analyses, and the possibility of in vitro cell culture. The system can analyze from 100 µL to 30 mL blood sample. The ParsortixTM reproducibility, high capture efficiency, and ability to produce highly enriched viable cells, has been validated by different groups. Lampignano et al. published a protocol to enrich, detect and

isolate EpCAM-negative CTCs from MBC patients, by combining potentials of both the ParsortixTM together with the automated micromanipulator CellCelectorTM. This workflow allows for further molecular characterization of CTCs such as the evaluation of the heterogeneity of PIK3CA mutational status within patientmatched EpCAM-high and EpCAM-low/negative CTCs in MBC patients [34].

ClearCell® FX1 (Clearbridge Biomedics) system is an automated cell retrieval system that allows the enrichment of CTCs from small amounts of blood in a relatively short time. This microfluidic biochip isolates CTCs based on size, deformability and inertia cell flow, relative to other blood components, by using inherent vortex flows present in their curvilinear channels, termed Dean Flow Fractionation (DFF). Through the process of DFF, blood cells separate and distribute themselves within the channels of the CTChip® FR1S (the chip inserted in the ClearCell® FX1 system), with the larger cells along the inner wall and the smaller cells away from it. As opposed to other microfluidic systems, ClearCell[®] FX1 requires a chemical red blood cells lysis. The intact CTCs are enriched in suspension, which allows for further molecular analyses and diagnostic assays. The technology is able to isolate viable CTCs allowing for an in vitro expansion of the cells as shown by Khoo et al., who were able to predict patient responses to therapy testing anti-cancer therapies on shortterm CTC cultures [35].

4.2.2.4 Dielectrophoresis

Dielectrophoresis (DEP) is an isolation technique based on physical properties that takes advantage of the distinct electrical properties of cells (dielectric properties). DEP relates to the movement of cells induced by electric field gradients since CTCs have a unique surface charge that distinguishes them from other cells. Thus, a dielectrophoretic flow field can be used to fractionate CTCs from blood cells based on their differential electrical properties.

ApoStream® (Apocell, Inc.) technology, for the isolation of CTCs based on the different dielectric properties (polarizability) of cells. The system can analyze from $50 \,\mu\text{L}$ to $10 \,\text{mL}$ of blood sample but need step-by-step operation by the user. It can be applicable for different cancer types including breast cancer. In a preliminary report, ApoStream® allowed the isolation of a heterogeneous population of both EpCAMpositive and EpCAM-negative CTCs in relation to the expression of EMT and stem cell markers, from the blood of patients with primary BC [36].

DEPArray™ (Menarini Silicon Biosystems) system. DEPArray[™] is usually a second purification step, used in combination with other enrichment methods (e.g. CellSearch®). This technology combines microfluidics and dielectrophoresis trapping individual cells in dielectrophoretic cages for latter recovery. Cells of interest are identified by image-based selection, isolated and recovered as single cells or pools of cells enabling downstream analysis. This technology has been successfully used in diverse clinical studies in BC. Notably, Mu et al. performed a genomic analysis which detected the TP53 R248W mutation from single and pools of CTCs by targeted sequencing on CTCs isolated from a patient with MBC, matching the mutation on patients' primary tumor [33].

4.2.3 Dual Combination Technologies

Despite the several technologies that have appeared in the market in recent years, it does not exist a technology either based on the physical or biological properties that can be applied as a standard for the isolation of CTCs. Therefore, in recent years, technologies combining both properties, immunoisolation and the different physical characteristics of CTC, have been developed to increase the efficiency in the isolation process.

The LiquidBiopsy® (Cynvenio Biosyntems, Inc.) platform relies in the immunomagnetic capture of CTCs on blood flow and it is performed on a chip that includes antibodies against EpCAM, Trop2, HER2, and MUC1/CD227. The system allows standard and customized assays. This platform achieves high target cell recovery and purity, and it enables downstream molecular characterization of rare cells and cell-free DNA (cfDNA) using NGS, FISH and immunohistochemistry. Analyses are performed for wholeblood samples. This system has been recently used for the detection of HER2-positive CTCs in BC, showing that these cells can be detected at all stages, including early BC, although detection rate was higher in metastatic patients [37].

Target SelectorTM (Biocept, Inc.) platform, has the capability to improve cell capture because it combines two steps, first the sample is passed through a blood collector tube for CTC and ctDNA isolation (CEE-SureTM) and next through a microchannel. The novelty is that this technology inhibits cell clumping and clogging of the microfluidic devices. The blood collection tube is designed to keep the sample at room temperature after blood collections from 5 to 7 days and to prevent blood coagulation preserving cells from lysis during storage or transportation.

IsoFlux (Fluxion Bioscience, Inc.), is based on microfluidics and immunomagnetic capture. The system combines the power of antibodybased magnetic bead separations with the precision of flow cytometry. The system allows the CTC recovery even during early-stage disease and it is applicable to different cancers such as breast, lung, colorectal, prostate, pancreatic, kidney, liver, bladder, etc. [38–42]. IsoFlux allows for CTC detection and enumeration as well as analysis through qPCR or NGS techniques. The system permits up to 4 analyses at the same time and processes from 7 to 10 mL of blood in less than 2 hours and a half.

The CTC-Chip is a dual technology that combines the use of microfluidics chips coated with antibodies for immunoisolation. The chip increases the sensitivity and the performance of the capture of CTCs from whole blood, using a smooth laminar flow that preserves the viability of 98% of the isolated CTCs. The chip is a silicon chip, about the size of a standard microscope slide containing an array of microposts with a specific geometric pattern coated with antibodies against EpCAM, allowing highthroughput capture of CTCs directly among posts. Staining can be performed to confirm CTCs origin and for enumeration or molecular characterization. The platform is flexible, since different antibodies could be potentially used to functionalize the microposts, resulting in the ability to detect a wide variety of CTC populations. The CTC-chip was shown to successfully identify CTCs in the peripheral blood of patients with MBC, among other cancers; as well as to capture cells EpCAM-low or EpCAM-positive with the same efficiency [43].

^{HB}CTC-Chip. The CTC-chip was further developed in a redesigned version, the herringbone-chip or "HB-Chip". The novelty of HB-Chip is its design that applies the microvortices generation to increase the number of interactions between target CTC and the antibody-coated chip surface. Its capability to isolate CTCs in patients with metastatic disease for different cancers was proved, as well as to isolate CTC clusters [44]. Due to its design, the low shear process allowed to detect clusters of CTCs difficult to detect by applying more aggressive technologies. Using this technology it was reported the presence of mesenchymal traits both in CTCs and CTC clusters from MBC patients, and the association of mesenchymal CTCs with disease progression [45]. In addition, using this technology to capture CTC clusters, Aceto et al. showed how the continuous presence of CTC clusters in the blood of metastatic prostate and breast cancer patients was associated with an adverse clinical outcome. Moreover, it helped to prove that CTC clusters are important oligoclonal precursors of BC metastasis [46].

CytoTrapNanoTM(CytoLumina Technologies). This is a technology in premarket validation. The concept of CytoTrapNanoTM is cell-affinity substrates with the ability to target a specific type of cancer cell due its morphology. It is a semi-automatic system and coupled with a microfluidic mixer is able to capture and quantify CTCs from a standard blood sample with a high level of sensitivity and specificity.

4.3 Single Cell CTC Isolation

Single cell isolation allows to analyze single cell molecular heterogeneity in a specific manner (without blood cells background). Three of the most popular technologies for single cell isolation base their approaches in the physical properties of the cells.

DEPArrayTM **system** (Menarini Silicon Biosystems), previously described (see Sect. 4.2.2.4).

VyCAP Puncher system (VyCAP B.V). The system allows the isolation of individual CTCs through specific hardware and software. It requires a pre-enrichment step of the CTCs (e.g. Cellsearch[®], ParsortixTM, RosetteSepTM, etc.) that then are sorted applying hydrodynamic forces to drag and distribute single cells in individual microwells of the isolation chip. After sorting, the chip is transferred to the Puncher system. This system allows imaging of the cells for the identification and recovery of individual CTCs in a fully automated manner for their subsequent molecular characterization. VyCAP is a versatile system since it also allows the capture and enumeration of CTCs by filtering cells according to size and stiffness.

Celsee Genesis system (Celsee diagnosis). This is a less known technology also applied for single cell isolation. It is a technology that bases the isolation in gravity forces and size-based exclusion allowing to capture individual cells into individual chambers in a microfluidic slide. The system allows for the capture and retrieval of CTCs for single cell downstream analysis.

4.4 Futures Perspectives in the Technologies Applied to CTCs Isolation and Their Clinical Application

Liquid biopsy is becoming an useful tool for the detection and management of breast cancer. In particular CTCs and ctDNA have gained remarkable attention as biomarkers. This is reflected in the increased number of technologies that have been patented for CTC isolation in the last decade. Despite so, the only one approved for its application in the clinical practice is the CellSearch®, which was patented more than 14 years ago. This shows the technical challenges that still nowadays need to be solved in order to take some other technologies into the clinic. In spite of the numerous clinical studies related to the analysis of CTCs in cancer patients and the proven usefulness of this biomarker, there are still certain technological limitations related to sensitivity and specificity. Currently, there is a lack of consensus regarding different methodological aspects about the isolation technique to be used, the type of sample, the conditions of collection or storage of the samples or the most suitable candidate biomarker to be used. However, it is anticipated that the clinical importance of CTCs will increase, especially in early stage cancers (when CTCs are present at extremely low frequencies) due to the dynamic development of techniques for the detection and analysis of CTCs, enabling prediction of disease progression. An effort should be made to improve the technologies allowing characterization of CTCs (in addition to enumeration) in order to obtain a clinical benefit in patients with early and advanced BC.

Given the growing interest of the clinical and scientific community on the information provided by the analysis of CTCs, technological advances are being made and large-scale clinical trials are underway. Although CTCs have great potential as biomarker for the diagnosis and prognosis of cancer, CTC enumeration has only informative application in patients with MBC, and enumeration alone does not offer suggestions on treatment selection or predict treatment failure. For this reason, technologies need to be validated in order to overcome the gap between the number of novel technologies developed and the number of them that enter the market and are being used in the clinical practice [47]. Reasons for this are the lack of standardized protocols for technologies validation, difficulty to access patients' samples for technology validation, and the high cost to manufacturing the technologies (takes a long time and money to build a manufacturing facility). Therefore, it is necessary a change on the strategy, and commercialization, as a final goal, should be taken into consideration by researchers from the initial steps of technology development. Moreover several reports have been published making comparison between the different isolation technologies assessing their performances with no clear conclusions, instead the technology of choice should be selected according to the purpose of each individual study, i.e. enumeration, downstream molecular analysis or cell culture [48, 49].

As previously seen, some technologies based only on epithelial markers recognition, fail to reflect all the potential CTCs subpopulations, e.g., EpCAM-negative or EpCAM-low cells [50]. Thus, technologies such as the gold standard, CellSearch[®], might underestimate those CTCs with the highest metastatic potential and more invasive phenotype, such as tumor cells that underwent epithelial-to-mesenchymal transition (EMT) or with stemnes features. Indeed, changes in EMT status of CTCs during treatments of individual patients with MBC have been reported, and a correlation between the number of EMT CTCs and therapeutic outcome showed [45]. Therefore, it is very important to elucidate which populations of CTCs are responsible of forming metastasis. The number of CTCs detected in MBC may increase if the EpCAM-high and EpCAM-low CTC populations were considered. A proof of this are studies showing improved CTC detection by employing EMT markers in addition to epithelial markers [51, 52]. However, we should not forget that only the presence of EpCAM-high CTCs correlates with poorer overall survival [53]. Antigen-independent approaches could eliminate the risk of underestimation of the different CTC populations; however, they could increase the isolation of a non- specific population. Although it is well defined that bigger cells mainly correspond to CTCs, there are technologies as Nanovelcro chips [54] or Epic CTC platform [55] that have shown how the presence of small nuclear CTCs correlates with the presence of visceral metastasis, mainly in prostate cancer patients. In this regard, isolation technologies based in the physical properties of CTCs are on

the rise in recent years, with microfluidic-based platforms having a prominent impact in the field.

Therefore, all these evidences highlight the importance of the molecular characterization of CTCs, as different subpopulations of CTCs may relate differently to the clinical outcome. The molecular characterization of CTCs could be instrumental to assess tumor heterogeneity, to predict site-specific metastasis, to detect treatment-resistant profiles and to identify new drug targets. In this sense, technologies allowing single CTC analysis are being put forward, as genetic information gathered from single cell analyses can be used for the accurate monitoring of cancer progression and treatment efficacy. Moreover, it may be useful for a precise selection of molecular prognostic and/or prediction markers, thereby improving the clinical outcomes of patients. Hence, studies are now focused on the molecular characterization of CTC with a clear clinical objective. There are several studies which demonstrate that molecular characterization of CTCs will provide useful information transferable to the clinic, but since different methods are applied both for enrichment and identification of CTCs, it is difficult to compare those results. The molecular characterization of individual CTCs with relevance in therapy has advanced a lot in recent years and it seems technically robust enough to be applied now in the clinical setting. However, detection and isolation of CTCs remains a challenge. In fact, not a single method of enrichment is able to collect each of the possible CTCs present in the bloodstream. In addition, no method will work with 100% efficiency, leading to loss of target cells and the isolation of unspecific cells. However, for treatment decisions it will be of utmost importance to improve CTC isolation efficiency to minimize the number of metastatic cancer patients reported with negative CTC-Test. In addition, an increased yield in CTC isolation will be relevant for the reproducibility of CTCs assays and for the analysis of the heterogeneity of CTCs. In this sense DLA allows for a more reliable detection of CTCs since when analyzing only a small part (around 2 ml) of the DLA product, a 2.5-fold increase in the detection

frequency is already observed and 30-fold in median CTC values [28]. This might allow to obtain CTCs in those metastatic patients who tested negative for CTCs in a 7.5–10 ml blood sample or have very few CTCs, for prediction and valid molecular diagnosis, respectively.

In addition, the use of diverse technologies and markers in the detection of CTCs has led to some discrepancies about the classification of a cell as a CTC (at phenotypic level). In this regard, new approaches are being developed as for example the ACCEPT software, a tool for automated CTC classification which was developed within the EU Cancer-ID project. It is an open source image analysis set that performs an Automated CTC Classification, Enumeration and PhenoTyping (ACCEPT). Zeune et al. showed how the ACCEPT image software allows a more reproducible quantification of CTCs analysis offering new fully automated and reproducible approaches. The study was done with a cohort of 132 MBC patients from whom blood samples were processed by CellSearch® and stained for HER2 expression. Images were digitally stored and were sent to six independent investigators to score the HER2 expression with and without ACCEPT. Concordance rate of the operators' scoring results for HER2 on CTCs was 30% and using the ACCEPT tool could increase to 51% [56].

Finally, all technologies developed for isolation of CTCs should fulfill some specific requirements on their performance. Thus, expert researchers in the field have suggested a standard set of performance criteria allowing the comparison and evaluation of technological platforms [30]. Assessment of these criteria, including aspects such as capture efficiency, purity, enrichment, throughput, cell viability, and release efficiency, will impact on the development of systems with a higher sensitivity and specificity, which will ultimately represent a benefit on the results of CTC detection studies. However, a major drawback on these studies is represented by the fact that when evaluating a system's performance for these parameters, blood samples from healthy donors "spiked" with known numbers of tumor cells from cancer cell lines are used, possible over-predicting the device performance. The reason is that samples from patients cannot be directly employed for this purpose since the actual number of CTCs in the sample is unknown. Thus, it is important to validate systems using clinical samples, and this is why these technologies are usually compared to the CellSearch[®], the only system cleared for the FDA for clinical use. Therefore, it is clear that there are still challenges that need to be solved in order to implement in the clinic the new technologies developed by researchers and companies.

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5

Advances in the Characterization of Circulating Tumor Cells in Metastatic Breast Cancer: Single Cell Analyses and Interactions, and Patient-Derived Models for Drug Testing

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Abstract

Metastasis is the major cause of breast cancer death worldwide. In metastatic breast cancer, circulating tumor cells (CTCs) can be captured from patient blood samples sequentially over time and thereby serve as surrogates to assess the biology of surviving cancer cells that may still persist in solitary or multiple metastatic sites following treatment. CTCs may thus function as potential real-time decisionmaking guides for selecting appropriate therapies during the course of disease or for the

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L. T. D. Chinen International Research Center, A.C. Camargo Cancer Centre, São Paulo, Brazil development and testing of new treatments. The heterogeneous nature of CTCs warrants the use of single cell platforms to better inform our understanding of these cancer cells. Current techniques for single cell analyses and techniques for investigating interactions between cancer and immune cells are discussed. In addition, methodologies for growing patient-derived CTCs in vitro or propagating them in vivo to facilitate CTC drug testing are reviewed. We advocate the use of CTCs in appropriate microenvironments to appraise the effectiveness of cancer chemotherapies, immunotherapies, and for the development of new cancer treatments, fundamental to personalizing and improving the clinical management of metastatic breast cancer.

Keywords

Circulating tumor cells (CTCs) · CTC culture · CTC-derived explants (CDX · also called CTC-derived xenografts by some authors) · CTC drug testing · CTC heterogeneity · Immunotherapy · Patient-derived xenografts (PDX) · Single cell analysis (SCA) · Single cell interactions

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5.1 Introduction

The mortality rate from breast cancer has declined over time, with improvements in screening, earlier diagnosis, and treatment [1-3]. However, although survival is increasing, the great majority of patients who progress to or are diagnosed de novo with metastatic breast cancer (MBC)which in the U.S. is projected to be over 168,000 cases in 2020-will eventually show resistance to sequential therapies over time and be the major cause of breast cancer death [4-7]. MBC is comprised of a molecularly heterogeneous group of tumors and diverse clinical presentations that influence survival patterns and treatment [7-10]. Thus, the search for better treatments for MBC continues, with continued need for real-world data and appropriate disease-relevant models for preclinical studies [11–14].

5.2 Tumor Heterogeneity

Heterogeneity is ubiquitous in human cancer. As tumors grow, genomic instability and environmental conditions-such as local oxygen concentrations, pH, local nutrients, mechanobiological factors, and immune cell interactions-may favor survival and clonal growth of distinct tumor cell subpopulations. Moreover, during multiple cycles of drug treatment, only cancer cells sensitive to a treatment are ablated, while drug-resistant cancer cell subpopulations survive [15, 16]. Consequently, tumors and their subsequent metastases may consist of individual cells with differing genomic composition, metabolism, physiology, and drug sensitivity. Genetic, epigenetic, protein and biomarker expression levels are commonly used to characterize tumor heterogeneity. Biomarker discordance has been documented between different regions of a primary tumor, between primary and metastatic tumors, and between different metastases [17–26]. Such discordances can limit selection of optimal therapy at any given point in the disease process.

Tumor heterogeneity is broadly classified as intertumoral (tumor by tumor) and intratumoral (differences within a tumor) heterogeneity. Intertumoral heterogeneity is a main barrier for cancer classification, and single-cell analysis plays a limited role in answering questions related to cancer classification. In contradistinction, intratumoral heterogeneity (ITH) is widely recognized as a barrier to overcome drug resistance and achieving effective cancer therapy.

5.3 Tumor Heterogeneity at Single-Cell Resolution

Single cell analysis (SCA) is uniquely powerful in resolving ITH and understanding tumor evolution [27]. ITH is a net effect of heterogeneity of malignant cells and diverse nonmalignant cells, such as immune cells, endothelial cells, and stromal fibroblasts [28]. Collectively, the tumor cells and associated nonmalignant cells comprise the complex tumor microenvironment (TME). To decipher the basic mechanism of drug resistance, metastasis, and immunotherapy response, it is essential to profile the heterogeneity of all cell types and states in the TME. Furthermore, it is essential to understand cancer-immune cell interactions [29] and immune response through various biomolecules such as cytokines [30].

Intratumoral heterogeneity has been widely studied by single-cell DNA [27, 31] and RNAseq methods [32–35]. SCA analysis using single cell RNA sequencing (scRNA-seq) has been used to study CTCs from patients with advanced breast cancer that lacked human epidermal growth factor receptor 2 (HER2) expression, showing a bimodal distribution of HER2+ and HER2- CTC subpopulations with an increasing fraction of HER2+ CTCs during disease progression; of note was that single cell growth of CTCs showed interconversion of HER2 status [32]. Miyamoto et al. studying gene expression profiles (using mRNA-seq) of 77 CTCs from 13 prostate cancer patients, noted heterogeneity in Wnt signaling pathways that could contribute to outcome of a therapy [33]. Using scRNA-seq, Patel et al. reported that individual tumors of primary glioblastoma contained a spectrum of subtypes and hybrid cellular states showing a

diversity of transcriptional programs, and this heterogeneity played an important role in glioblastoma biology, prognosis, and therapy [34]. Tirosh et al. profiled the multicellular ecosystem of metastatic melanoma by scRNA-seq [35]. In this work, the authors noted that the tumor cells displayed transcriptional heterogeneity associated with the cell cycle, spatial context, and a drug-resistance program, while the nonmalignant immune cells displayed dynamic connection between T cell exhaustion and activation, and heterogeneity was reported across 19 patients. There have been numerous studies analyzing mutation evolution and gene expression profiles of various tumors. However, the cellular processes and function, such as immune response, depend on the expression level of proteins. For most of the genes, there is a poor correlation between mRNA expression level and corresponding proteins [36] or cytokines [37]. Compared to RNA-seq studies, there have been limited studies analyzing single-cell protein expression in the context of heterogeneity. This is primarily because flow cytometry allowed profiling of limited numbers of proteins (< 10). Introduction of cytometry by time of flight (CyTOF®) [38] technology and Imaging Mass Cytometry [39] (IMC) enabled analysis of 32 proteins and protein modifications. CyTOF was used to elucidate ITH in acute myeloid leukemia (AML) [40]. The authors reported that surface phenotypes and regulatory intracellular signaling are decoupled in leukemia. Single cell protein profiling of cancer and immune cells have been applied to other cancer types such as renal cancer [28], acute lymphoblastic leukemia [41], ovarian carcinoma [42], hepatocellular carcinoma [43], and lung adenocarcinoma [44].

To further understand the complexity of ITH, development of technologies that can profile multi-omics such as genome, epigenome (chromatin accessibility, methylation), transcriptome, proteome, and secretome (cytokines) simultaneously per cell would be required. Recently, Gkountela et al. profiled DNA methylation patterns in circulating tumor cells from breast cancer patients and xenograft models [45]. The authors reported that hypomethylation profiles of CTC clusters correlated with poor prognosis in breast cancer, and disruption of CTC clusters reverted the methylation profile and suppressed metastases. There also has been limited work on multi-omics profiling of the TME [46, 47]. Bian et al. profiled somatic copy number alterations, DNA methylation, and transcriptome simultaneously through a single-cell triple omics sequenctechnique. ing (scTrio-seq) The authors demonstrated the feasibility of reconstructing genetic lineages based on epigenetic and transcriptomics signatures [46]. Rodriguez-Meira et al. reported a novel method called TARGETseq that combines genomic DNA and cDNA genotyping with single-cell RNA-seq [47]. Further development of multi-omics techniques will enable profiling of cytokines and other -omics at single cell resolution.

For patients with multifocal metastatic disease, tissue biopsy may be impractical or risky and, if metastatic biopsy is performed, it is generally not repeated as metastases grow or new metastases develop. Using CTCs from blood draws allows live cells shed from metastases in multiple sites to be interrogated as surrogates of the spectrum of surviving cancer cells in metastatic disease. Using high dimensional single cell transcriptional profiling, we have shown that individual CTCs from patients with primary and metastatic breast cancer are heterogeneous, even within a single blood draw, and distinct from single cells from cancer cell lines used for drug discovery [48]. It is likely that sequential SCA investigations of CTCs through the course of disease may offer insight into more optimally tailored regimens, revealing markers or signaling pathways that may suggest unexpected therapeutic approaches.

5.4 Single Cell Interactions

New platforms to study single-cell RNAsequencing have enabled the detection of cellcell interactions, delving more deeply into ligand-receptor (L-R) interactions and its effects on gene expression [49]. Considering the importance of the development of new cancer therapies, research groups are seeking to better understand characteristics that define the interaction between cancer and immune cells. While interactions between cells within the primary tumor and its microenvironment are often studied, the role of immune cells and their interaction with tumor cells during cancer dissemination may be equally if not more important [50]. As precursors of metastasis and when isolated from blood, CTCs may be found to be associated with white blood cells (WBCs), tumor-derived fibroblasts, and/or endothelial cells [51, 52], interactions that may modify cell programs. In another interaction model, a component of innate immunity (macrophages) was described promoting incongruously aggressive pro-tumorigenic behavior when stimulated by an immune checkpoint inhibitor [53].

Transcriptional profiles present in individual malignant and non-malignant cells within a metastatic melanoma tumor were studied using scRNA-seq and t-distributed stochastic neighbor embedding (t-SNE) plots to define different transcriptional states associated with different spatial locations within a tumor and presence of various neighboring immune and non-immune cells [35]. scRNA-seq was used to study ligand-receptor interaction pattern across different immune cell types and tumor cells, particularly chemokine interactions [54]. Chen et al. developed a microchannel plate with three-dimensional (3D) concave microwells for growing liver tumor spheroids and co-culturing them with hepatic stellate cells. Co-culture studies accompanied by drug testing showed recapitulation of epithelialmesenchymal transition (EMT) and chemoresistance that suggested its use for not only cell-cell interaction studies, but also for drug response testing [55]. We have been using a single cell microfluidic platform that allows single cell selection, cell-cell interactions, drug perturbations, and on-chip preparation for RNA-Seq analyses (PolarisTM, Fluidigm Corporation) to investigate single tumor cell-immune cell interactions, focusing on the role of NK cells in antitumoral activity (Fig. 5.1).

Another interesting approach is to evaluate both transcriptome and protein profiles simulta-

neously following cell-cell interactions. Each cell may be labeled and then measured following an interaction, targeting each cell with appropriate barcoded probes and then incubating both cells together. CITE-seq (cellular indexing of transcriptomes and epitopes by sequencing) [56] and REAP-seq (RNA expression and protein sequencing assay) [57] are similar techniques that use DNA barcodes attached to antibodies, enabling the discovery of multi-omic interaction effects [58]. Different mass spectrometry methodologies may be applied to study single-cells and the biology of cell-cell interactions [59, 60]. A method using high-throughput protein analyses is mass cytometry, allowing measurement of about 40 proteins simultaneously in single cells. In this technique, target cells are labeled with multiplex metal-conjugated antibodies, and the target protein abundance are detected using CyTOF mass spectrometry [61]. Another study using imaging mass cytometry has shown activated signaling pathways spatially distributed among heterogeneous subclones of triple-negative breast cancer and the effect of therapeutics on signaling pathway activation patterns and subclonal communication with other subclones and the TME [62].

Epigenetic alterations that may occur after in vivo interactions can be profiled by Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) [63]. This method identifies gene regulatory elements through transposition of sequencing adaptors in regions where the chromatin is accessible. Further extension of this method led to ATAC-see, which enables profiling of spatial organization of the accessible genome. ATAC-see utilizes optimized Tn5 transposases with fluorescent adaptors to profile open chromatin structure in single cells fixed on a substrate [64].

One of the approaches of monitoring in vivo cell-cell interaction is a strategy that uses ligandinduced intramembrane proteolysis. This has been shown with glial cells and neurons in transgenic Drosophila, based on the Notch-Delta interaction mechanism which controls cell fate during fly development through cell-cell interactions [65]. However, this strategy can only be used to investigate cell-cell interaction for Notch-



Fig. 5.1 Single cell analysis and cell-cell interactions analysis. Single cell analysis or single cell interactions between individual tumor cells (including CTCs) and immune cells may be assayed to evaluate anti-tumoral activity, differential gene expression, or evaluation of protein and/or metabolic markers. (a and b) microfluidic devices or small chamber well plates may be used to perform single cell analysis or cell-cell incubations; (c1) cells

Delta signaling pathways. Another important interaction to investigate is the effect of myeloidderived suppressor cells (MDSCs) on cells of the immune system with downstream consequences on tumor growth and spread. MDSCs target T cells, which then suppresses the immune system. Some proteins involved in MDSC immune suppression mechanisms include arginase (ARG1), inducible nitric oxide synthase (iNOS), transforming growth factor beta (TGF β), interleukin 10 (IL10), cyclooxygenase-2 (COX-2), indoleamine 2,3-dioxygenase (IDO) sequestration of cysteine, and decrease of L-selectin expression by T cells [66]. There is also evidence to suggest that MDSCs interact with the innate immune system and modulate the activity of macrophages, dendritic cells (DCs) and natural killer (NK) cells [67]. Recent in vivo studies in breast cancer patients have shown that polymorphonuclear-MDSCs (PMN-MDSCs) interact with CTCs in heterotypic clusters; these PMN-MDSCs induce pro-survival responses in CTCs, and in xenograft models, these interactions enhance metastasis formation [68]. Tumor-educated platelets (TEPs) interact with tumor cells to influence tumor growth and dissemination [69]. This interaction affects both the expression of genes in tumor

are lysed, mRNA is reverse transcribed, cDNA is preamplified, and the library is prepared and sequenced; other techniques may be used to study protein expression: immunofluorescence (c2), DNA-barcoded antibodies (c3), mass spectrometry (c4), or mass cytometry (c5); (d) bioinformatic analyses are then performed, such as by principal component (PC) analysis and multiple other techniques. NGS = next generation sequencing

cells and the RNA (coding and non-coding) profile of blood platelets [70]. Still under investigation are the mechanisms involved in platelet education and how different platelet subpopulations change in cancer patients. TEP RNA biomarkers may contribute to the liquid biopsy field through easier disease monitoring or even earlier detection [71]. This interaction may also serve as a treatment target, as discussed in a later section.

5.5 Reason for Propagating CTCs

The clinical relevance of available preclinical models is frequently debated due to problems such as lack of genetic heterogeneity, transcriptomic drift, cross-contamination, and whether they are indeed representative of the patient being treated [72]. Thus, the development of appropriate and clinically-relevant cancer models is critical. CTCs are critical effectors of cancer metastasis, but their numbers are limited. Moreover, the process of growth and expansion of CTCs in culture still remains challenging owing to their rarity and low viability [73]. Variability in morphological, molecular, and functional aspects due to genetic heterogeneity

further adds to this conundrum [74]. Thus, strategies for the expansion of CTCs may shed light on the molecular signature and biology, including metastatic homing mechanisms, of the parent tumor or tumors. Due to their rarity, the propagation of CTCs holds promise for establishing patient-specific preclinical models for accurate genetic and phenotypic evaluation and for testing preclinical efficacy of various drugs or drug combinations [75]. Notable progress has been made regarding the isolation and in vitro propagation of CTCs from the peripheral blood of cancer patients. Recent studies have shown the possibility of developing 2D and 3D (tumor spheroid or organoid) cultures that closely relate to the CTCs from which they were derived.

Organoid cultures are grown in a 3D environment and are emerging as a novel preclinical model to understand the structure and function of the organ sample from which they originate. When tumor tissues are isolated from a patient and cultured, these tumor organoids are able to partially mimic the complexity of the original tumor [76]. When tumor tissues are cocultured with immune cells and tumor-associated stromal cells, the patient's tumor tissue phenotype may be sustained, allowing therapeutic responses to different drugs to be effectively studied [77]. This ex vivo model has become a crucial tool in the emerging field of personalized medicine. Sachs et al. successfully prepared more than 100 mammary epithelial tumor organoid lines from primary and metastatic breast cancer patients. These organoids typically mimicked the micro-anatomy of the original tumor, including the hormone receptor and HER2 status of the original tumor. Importantly, therapeutic response of organoid cultures to tamoxifen, when determinable, showed a match between the in vitro response and the therapeutic response of the patients from whom the organoids were derived, as would be expected for an in vitro surrogate of a patient's breast cancer; similarly, comparing drug response in xenograft models generated in mice implanted with organoids grown from patient tumors, the in vivo response of the mice to drugs blocking the HER2 signaling pathway generally matched the in vitro response of the organoid culture [78]. In a separate study designed

to systematically assess T cell-mediated tumor recognition, tumor organoid cultures positive for major histocompatibility complex (MHC) class 1 from non-small-cell lung cancers and mismatch repair-deficient colorectal cancers were cocultured with peripheral blood lymphocytes from the same patients to assess the activation of T cell response against tumor cells. They indeed showed that tumor-reactive T cells were induced by coculture, and that further co-culture of tumor organoids with these autologous tumor-reactive T cell populations caused apoptosis and reduced survival of the tumor organoids [79]. Patient-derived 3D organoid lines from patients with advanced prostate cancer were successfully developed from bone and soft tissue metastases, a pleural effusion, and, in one case out of 17 blood samples with CTC counts greater than 100 in 8 ml of blood, a CTC organoid line from a patient with castration resistant prostate cancer (CRPC); the organoid lines and subcutaneous xenografts made from the organoid lines recapitulated the histopathological and molecular features of the original samples, reflecting the diversity of genomic, transcriptomic, and protein expression features found in CRPC, and showed expected results during drug testing [80].

Growing such cultures ex vivo facilitates intervention by chemotherapeutic drugs and also its interactions with immune cells, which can be monitored and studied in real time. These cultures can further be readily integrated into in vivo studies, either by orthotopically or subcutaneously injecting them into immunocompromised mice to establish a CTC-derived mouse xenograft [81]. In a different approach, CTCs isolated by negative enrichment from the peripheral blood of patients can be tumorigenic after direct implantation into mice to establish CTC-derived explants (CTX) [82]. These ex vivo models exploit the potentially invasive nature of CTCs and serve as emerging preclinical models for patients with invasive cancers. Ex vivo expansion of CTCs by culturing of CTCs in vitro, both short-term and long-term, and in vivo growth of CTCs from patient blood samples are exciting approaches for investigations into the biology and treatment of breast cancer.

5.6 In vitro Expansion

5.6.1 Short-Term Culture

CTCs from patients have been propagated in vitro by multiple groups for various types of cancer [83–93]. Short-term cultures of CTCs vary from a few days to a few months depending on the type of experiments. These short-term cultures have been mostly utilized for karyotyping, immunohistochemical analysis, cytomorphological analysis, genomic profiling, gene expression profiling and proteomic profiling. Short-term culture may be more closely related to the malignant cells of the tumor as longer term tumor growth may accumulate genetic or phenotypic changes through prolonged passaging. Short-term ex vivo expansion of CTCs from breast cancer patients has also been established. In one study, CTCs from six patients were cultured for 16-18 days, and contained heterogeneous populations of cells, with epithelial cell adhesion molecule (EpCAM)positivity of cultures from each patient ranging from 35% to 86%. The cultures were then analyzed using a panel of genetic mutations and compared with those of the primary tumor; the similarity of mutation profiles also demonstrated the suitability of the CTC cultures as in vitro surrogates for breast cancer molecular studies [94]. Another group isolated CTCs using a size-based filtration membrane and cultured them briefly for 3-5 days for use in downstream molecular analyses and monitoring patient response to different therapeutic regimes in different types and stages of breast cancer [95]. Using the same size-based and antigen-independent membrane filter technique, CTCs from 167 breast cancer patients were either analyzed immediately or cultured in vitro by placing the filter in a 6-well cultivation plate for a minimum of 14 days, facilitating immunocytochemical as well as downstream molecular analyses by qPCR. In some patients, expression status of HER2 and estrogen receptor (ER) in CTCs differed from that of matched primary tumors, and over time in multiple different blood samples, HER2 status change of CTCs was bidirectional, with only unidirectional change in ER status (ESR+ to ESR-) [96].

Pizon et al. isolated a variable fraction of circulating cells from breast cancer patients based on EpCAM expression and grew those with high-CTC counts as tumor spheroids, culturing them up to 28 days. CTCs isolated from different patients were heterogeneous and when examined individually, showed variable expression of nanog and vimentin; the ability to grow as tumor spheroids appeared to correlate with tumor aggressiveness [97]. A similar study used a functionally-based approach to isolate breast cancer CTCs by enriching for an invasive subpopulation of CTCs using collagen adhesion matrix (CAM) assay and, using gene expression analysis, identified variable CTC populations with epithelial lineage, tumor progenitor cells with stem/invasive cell properties, and mixed epithelial/progenitor phenotypes; CAM-enriched CTCs were also capable of growing in culture on the CAM scaffold for up to 33 days [98]. A related work used fluorescence-activated cell sorting (FACS) to isolate CTC subsets from the blood of breast cancer patients with and without brain metastases and cultured them as 3D CTC spheroids for up to 30-40 days. Prior to culture, EpCAM-negative CTC subpopulations were selected for CD44+/CD24- cells, related to stemness, and then selected for urokinase plasminogen activator receptor (uPAR) and integrin beta-1 (int β 1) positivity, related to breast cancer dormancy. These distinct molecular attributes allowed the CTCs to form spheroids and grow in culture for use in identifying patients at risk for forming brain metastases [99]. Khoo et al. also were able to establish in vitro cultures of CTCs for 2-8 weeks from breast cancer patient blood using special laser-ablated microwells. Cultivated cells were stained with multiple markers to determine cell composition over time, including markers for leukocytes (CD45 and CD18), hematopoietic precursors (CD34), monocytes (CD14 and CD16), megakaryocytes (thrombospondin-1), and endothelial cells (CD31 and von Willebrand factor, VWF). After 2 weeks, cultures consisted mainly of three cell types: 1) CTCs that expressed cytokeratin but not CD45 (CK+/CD45-), 2) macrophages that expressed migratory inhibitory factor (MIF) and CD68, and 3) NK

cells that expressed CD56. The CD45 negative generally were cells either small cells $(\leq 25 \text{ microns with a high nuclear/cytoplasmic})$ ratio) corresponding to CTCs and large cells (>25 microns with a low nuclear to cytoplasmic ratio) comprised of macrophages and NK-like cells. The fraction of stem-like cells in CTC cultures was also increased by cultivation under hypoxic conditions (1% oxygen). The CTCs grown for 2 weeks contained heterogeneous groups of cells expressing both epithelial (pan-CK) and mesenchymal (vimentin) markers, confirmed by RNA fluorescence in situ hybridization using nine epithelial genes and four mesenchymal genes (PTX3, SERPINE2, VIM, and FASCIN). Moreover, the formation of CTC clusters was inversely correlated with treatment duration and the persistence of CTC cluster formation appeared predictive of lack of response to anticancer therapy [100].

5.6.2 Long-Term Culture

We define long-term culture of CTCs as cultures that can be maintained for greater than 6 months. Culturing of CTCs isolated from peripheral blood of breast cancer patients is challenging, with few reports of long-term culture. The first successful attempt at cultivating CTCs as a continuous culture was done by Zhang et al. in 2013. They established three CTC lines (CTC-1, CTC-2, and CTC-3) from metastatic breast cancer patients. CTCs captured by FACS using the molecular pattern EpCAM-/ALDH1+/CD45- were able to grow continuously and form cell lines, while CTCs selected using EpCAM+/ALDH1+/CD45were not able to survive in culture for more than 2 weeks. Intracardiac or tail vein injection of these three cell lines into nude mice produced brain and lung metastases for CTC-1 only, whereas the other two CTC lines formed only lung metastases. Further, after expansion of these three EpCAM-negative cell lines, selection by FACS for CTC subpopulations that expressed HER2, epidermal growth factor receptor (EGFR), heparanase (HPSE), and Notch 1 (EpCAM-/ EGFR+/HPSE+/Notch1+, known as brain

metastasis selected markers, BMSM) produced cells capable of homing to and forming brain metastases. All three new CTC cell lines had brain metastatic potential and were capable of generating brain and lung metastases in nude mice [101].

Subsequently, other laboratories were able to successfully culture CTCs long-term. In one study, six CTC cell lines were derived from patients with metastatic ER-positive breast cancer whose disease was progressing on therapy (BRx-07, BRx-33, BRx-42, BRx-50, BRx-61, and BRx-68). These oligoclonal CTC cultures were cultivated from microfluidically-captured CTCs and grown as tumor spheres in serum-free media supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (FGF) under hypoxic conditions (4% oxygen), with continuous growth in vitro for over 6 months. However, unlike the other five ER-positive CTC lines, BRx-07 did not retain its original ER expression in vitro. Of these six cell lines, three (BRx-07, BRx-61, and BRx-68) were able to develop tumors in NOD scid gamma (NSG) mice, depicting in vivo tumorigenicity, and with BRx-07 regaining ER expression in vivo [81]. The first report of a CTC-derived cell line established from colon cancer was by Cayrefourcq et al. and was named CTC-MCC-41. After examining blood from 71 patients with metastatic colorectal cancer (CRC), 50 patients were identified whose blood sample had at least one detectable CTC, and three had greater than 100 CTCs. Two of these high CTC number blood samples produced CTC cultures that propagated for at least 2 months when initially incubated under hypoxic conditions (2% oxygen); however, only one developed into a permanent CTC cell line, derived from a patient with rapidly progressive metastatic CRC that was unresponsive to multiple therapies, with that CTC line still growing under normoxic conditions for more than 16 months at time of publication. CTCs were isolated by negative selection and grown as tumor spheres in non-adherent conditions. This CTC line was also expanded as a CTC-derived xenograft following subcutaneous injection into SCID mice. Interestingly, the CTC cell line expressed

stem cell-like markers and an intermediate epithelial-mesenchymal phenotype that may have added to its ex vivo growth advantage. Further, KRAS and BRAF mutational status and CK20 expression were preserved between the CTC cell line, the original patient tumor tissue, and xenograft tumor tissue, indicating suitability for a personalized medicine approach for testing future drug therapies [102]. In addition to CTC-MCC-41 described above, eight more permanent CTC lines were generated from sequential blood draws from the same patient during progression of that patient's metastatic CRC, and thereby facilitating further study of clonal selection in metastatic cancer. Notably, this newer paper describes that these remain the only CTC cell lines derived from this one patient after testing blood samples from 168 patients with metastatic CRC [103]. As also described, Gao et al. were able to make an organoid line from CTCs isolated from a patient with castration-resistant prostate cancer. The organoid line, MSK-PCa5, was established from a patient who had a CTC count of >100 cells per 8ml of blood. These CTCs were cultured as organoids in Matrigel (BD Biosciences) with reduced growth factors. Further, it formed tumor when it was injected as a subcutaneous xenograft in a SCID mouse [80]. In another report, a CTC-derived cell line (CTC-3) was established and characterized from the blood sample of a patient with metastatic ER-positive breast cancer. These cells had high nuclear/cytoplasmic ratio and were able to form spheroids [104].

Our collaborative group has previously shown that some of the drivers for the immortalization of cells include *hTERT* expression, telomerase activity, downregulation of genes associated with TGF β signaling, and overexpression of oxidoreductase genes [105, 106]. Studies have shown that additional genetic and phenotypic changes are acquired when stable cell lines are generated from the patient-derived samples [72, 107]. However, studies have shown that continuous cultures from CTCs retains the important genetic features of the patient's tumor [80, 102].

5.7 In vivo Expansion

In addition to the short-term and long-term in vitro culture of CTCs, in vivo platforms have also been used for their expansion. Breast cancer is a highly heterogeneous disease both intertumorally and intratumorally, as previously discussed, and there can be significant clonal diversity within a patient's tumor. Inconsistency between xenograft studies from a diverse array of cell lines and individual patients' tumors may be bridged by patient-derived xenograft (PDX) models. Such models are generated by the implantation of freshly resected cancerous tissue from a patient's tumor either subcutaneously or orthotopically into an immune-deficient mouse. Conceptually, PDX models maintain the complex tumor heterogeneity by preserving the crucial molecular properties of the original tumor and by providing associated TME when implanted as tumor fragments (although mouse stroma will eventually replace human stroma by the second passage in PDX models [108]). PDX models also provide a renewable source of original patient tumor for interrogation with diverse targeted therapies and new drug development, in contrast to the clinical setting. However, because the tumor growth time of some PDX models (often 2-8 months or more), this may or may not be of benefit the specific patient from which it was derived and instead benefit future patients with molecularly similar tumors. Zhang et al. showed the response of mTOR inhibitors in a panel of seven triple-negative breast cancer (TNBC) patient-derived orthotopic xenograft (PDOX) models, representing four different molecular subtypes of TNBC, and with all histologically and genomically matching original patient tumors [109]. In breast cancer PDX research, a consortium of academic researchers worldwide has curated over 500 stably transplantable breast cancer PDX models and their information, representing three major clinical subtypes of breast cancer, estrogen receptor positive (ER+), HER2+, and TNBC [110]. PDX models (also called patient-derived tumor xenografts, PDTX) and

short-term culture of cells from PDTX models (PDTX-derived tumor cells, PDTCs) are both platforms that offer clinically relevant options to guide the testing and development of drug therapies for individualized breast cancer management [111]. PDX models provide a lot of promise in the field of precision medicine but also require protocol standardization for tissue collection, tracking and handling, and the propagation of the primary tumor from patient to mouse as well as further growth of xenografted tissue in 3D culture [112]. PDX models can also be a useful source of CTCs for in vitro interrogations. Our lab and others have shown that PDX models of breast cancer are able to shed CTCs and metastasize to distant organs; these CTCs can be then used for downstream molecular investigations using immunofluorescence, flow cytometry, real-time quantitative reverse transcription-PCR (qRT-PCR), and single cell gene expression analyses [113–118].

Tumor samples used to generate PDX models are derived from patients at the time of surgical excision or needle biopsy of a tumor. Both approaches limit the use of PDX models to track temporal changes that tumors undergo following treatment and during disease progression. However, blood samples (i.e., liquid biopsy) offer an easy and minimally invasive approach for obtaining patient tumor material serially and in real-time. CTCs isolated from the peripheral blood of patients may be grown in immunocompromised mice to generate CTC-derived explants (CDXs) [119]. CDX models recapitulate the molecular characteristics and heterogeneity of patient tumors to shed light on metastatic biology and, importantly, for use as preclinical models for drug testing and drug development.

The first successful attempt to create CDX model was done by Hodgkinson et al. from patients with metastatic small-cell lung cancer (SCLC), which unlike most solid tumors, shed hundreds to thousands of CTCs. They implanted negatively enriched CTCs from both chemosensitive and chemorefractory SCLC patients; regardless of the therapeutic responsiveness, CDX models were successfully generated when CTC numbers were greater than 400 per 7.5 ml of

blood. Genomic analyses between the isolated CTCs and tumor from the CDX models showed similar molecular signatures. Interestingly, the CDX models perfectly recapitulated the original patient's response to platinum and etopside treatment, proving the possibility of predictive tailored therapy on patients [82]. Since then, many studies have reported successful attempts to propagate CTCs in vivo through CDX models in lung cancer [120–122], melanoma [123], and breast cancer [124, 125]. CDX (also used to denote 'CTC-derived xenografts' by other authors) tumor cells have also been subsequently propagated in vitro in short-term culture (up to 5 weeks) and have shown similar drug sensitivities, thereby facilitating in vitro drug screening [126].

Other studies have used long-term cultured CTCs from the breast cancer patients to form xenografts [81, 101]. But there are few studies that have directly isolated CTCs from breast cancer patients to make CDX models because CTC numbers are generally low (single digit to double digit range), even in metastatic breast cancer. Baccelli et al. developed CDX models from a metastasis-initiating cell population among CTCs isolated from primary human luminal breast cancer patients. These CTC subpopulations, which were EpCAM+CD44+CD47+ MET+, were injected into the femurs of NSG mice, a bone marrow compartment that potentially represents a privileged hematopoietic stem cell niche, and then were able to grow and disseminate, forming multiple lung, liver, and bone metastases. However, it was observed that only when over 1100 CTCs were transplanted into the femur did successful xenografts occur [127]. In another study, the CDX models were developed in NOD/SCID mice where CTCs positive for M30 and HER2 were isolated from metastatic breast cancer patients. It was interesting to observe that only approximately 200 and 400 CTCs were injected into the mice that successfully formed metastases in spleen and bone marrow. Further, they were able to detect CTCs in the mouse peripheral blood [128]. In a recent study, Pereira-Veiga et al. successfully made CDX models from CTCs isolated from a patient with

metastatic TNBC whose CTC count was 969 CTCs/7.5 ml blood and included 74 CTC clusters of 2-7 cells. A nude mouse was injected subcutaneously with negatively enriched CTCs, and after tumor growth, the xenograft tumor was split: a portion was implanted subcutaneously in a Scid Beige mouse and another portion was disaggregated and cultivated in vitro for 2 weeks and then injected orthotopically into the mammary fat pad of another Scid Beige mouse and tumor growth was monitored. CTCs from mouse blood were also detected. Gene Ontology (GO) analysis on CDX tumors and matched patient tumor and lymph node metastases indicated WNT signaling pathway and genes associated with cell cycle were crucial to TNBC tumor progression [124]. Vishnoi et al. successfully created a TNBC CDX model that specifically formed liver metastases. They used a negative depletion strategy to isolate a CD45-/CD34-/CD105-/CD90-/CD73- cell population from TNBC patients that were enriched for CTCs. When these CTCs were injected by an intracardiac route into NSG mice, about 66% of them developed liver metastases. They then sequentially propagated the metastatic liver tumor for four generations using the cells from the liver tumor to determine a TNBC liver metastasis gene signature as well as identify six candidate drug target genes for the development of new therapeutics [125]. CDX models can be further used as the sources of CTCs or patient's tumor cells, which again can be interrogated for metastatic research studies. In addition, human cell line-derived tumor xenograft models [129] and syngeneic and transgenic mouse models [130, 131] using established cancer cells lines have been previously used to isolate, culture, and propagate CTCs, offering insights into the relation between hypoxia and CTCs and other biological insights.

All the above studies point toward continued sources of patient CTCs or tumor cells expanded ex vivo that can be used for novel therapeutic targeting along with the multi-omics analyses that can provide a large array of data for biomarker and drug screening in cancer and for use in investigating metastatic biology, as depicted in Fig. 5.2. These ex vivo preclinical tumor models preserve the original molecular characteristics of the parent tumor tissue or CTCs and should prove useful for advancing personalized medicine.

5.8 Drug Testing Using CTC Models

Breast cancer is still the leading cause of cancer death in women worldwide and the second leading cause after lung cancer in North America, Northern Europe, and Australia/New Zealand [132]. There are diverse molecular phenotypes of breast cancer based on gene expression profiling, corresponding pathology biomarkers, and integrative cluster groupings based on genetic fingerprinting and genomic copy number drivers [133, 134]. However, intratumoral heterogeneity and spatiotemporal heterogeneity among different metastases, including frequent acquisition of driver mutations in distant metastases not identified in the primary tumor, will impact the effectiveness of therapeutic drugs that may only target tumor subclones with specific genetic aberrations, inferring a need for new therapeutic strategies for treating metastatic breast cancer [135–137].

CTCs and CTC-derived preclinical models offer solutions for studying tumor heterogeneity and molecular changes over time, thus helping guide, develop, and test new therapeutic strategies against breast cancer. The characterization and monitoring of CTCs may offer insight into the molecular landscapes of a patient's tumor in real-time and help monitor tumor growth and therapeutic response [138]. Many prospective studies contribute to the efficacy of chemotherapy in breast cancer by monitoring the CTCs from blood biopsies [139–142].

CTCs may themselves be utilized as therapeutic targets. Novel methods of targeting CTCs, such as by incorporating synthetic microparticles containing apoptosis-inducing substances into CTC microemboli, thereby using them as a "Trojan Horse" for delivering therapy,



Fig. 5.2 Ex vivo propagation of CTCs from patients and patient-derived models. Tumor fragments or cells from a patient's primary breast cancer or metastasis may be directly implanted or inoculated into the mammary fat pad of immunocompromised mice to generate a patientderived xenograft (PDX) model; human CTCs isolated from PDX mouse blood may then be propagated by in vitro cell culture. CTCs from a patient blood sample may also be directly propagated by in vitro culture and

then inoculated into immunocompromised mice; alternatively, CTCs from a patient may be isolated by positive or negative selection and propagated in mice as CTC-derived explants (CDX, also called CTC-derived xenografts). These ex vivo models may ideally be used for drug testing to predict therapeutic responses of patients or to perform multi-omic, immunohistological, and immunohistochemical analyses for elucidating metastatic biology and identifying new targets for drug discovery

have been reported to decrease lung metastases in a mouse model [143]; interruption of CTC/ platelet interactions is another strategy under investigation [144].

CTCs may also be used to identify drug sensitivities of breast tumors [145]. Yu et al. derived CTC cell lines, growing for greater than 6 months, isolated from the blood of patients with metastatic ER-positive breast cancer who were off treatment or progressing on therapy. Cell lines were generated by 3D cultures (tumor spheres) under hypoxic conditions in 6/36 patients. CTC lines and CDX models generated from some CTC cultures were tested for response to an array of anticancer drugs that included inhibitors of PI3K, CDK4/6, IGFR, ER, mTOR, HSP90, FGFR, PARP, and some first-line chemotherapeutic drugs like paclitaxel, capecitabine, and doxorubicin. In this proof of concept study, these drugs alone or in combination targeted CTC-

derived cell lines both with mutated oncogenic drivers like PIK3CA, FGFR2, TP53, ESR1, and BRCA2 or non-mutated targets like HSP90 and IGFR. Some of the drug sensitivity and resistance results were concordant with available clinical histories of the patients, and combination treatments that targeted two pathways were more effective than single drug treatment in some cell lines and CDX mouse models tested with specific oncogenic driver mutations [81]. Another study described the development and testing of a special microfluidic platform designed for growing non-enriched CTCs in short-term culture (within 2 weeks) and then performing on-chip drug screening, finding that co-culture with immune cells promoted cluster formation and CTC expansion. Importantly, the ability to form clusters was inversely correlated with drug concentration and in vitro drug sensitivity, suggesting its use as a CTC drug-screening assay [146, 147].

5.9 Future Perspectives

Patient-derived CTCs can now be cultured ex vivo as short-term cultures or long-term cultures, and can be available for expansion or retransplanted into immunocompromised murine models as per the requirement of the experiments. While some researchers have been able to establish continuous cell lines with patient CTCs, success rates remain relatively low and these ex vivo culture methods still require further refinement and optimization for regular use in laboratory protocols or clinical applications. As the TME encompasses multiple cell types, biochemical signals, extracellular matrix, varied oxygen levels, and also mechanical stress and tensions that drives towards metastasis, creating biomimetic organ microenvironments or strategies involving the metastatic niche could provide cues for enhancing CTC growth ex vivo [148, 149, 150]. Mimicking these microenvironment by 3D cultures using biomaterials, bio-scaffolds, cytokines, immune cells, and tissue-specific cells on microfluidic platforms could be utilized to create a physiologically relevant cancer model [149]. Such 3D cultures could be manipulated and studied to elucidate the dynamics of TME interaction with CTCs during metastasis formation and growth and also used for developing and testing therapeutic approaches against metastatic breast cancer [148]. Such co-clinical approaches are expected to be used widely in therapeutic development where assessments of CTCs and CDX models can be directly correlated with patients' treatment and clinical outcomes [119, 151]. This allows the evaluation of real-time response to different therapies through disease evolution. However, these strategies have been plagued by the pertinent problems of cultivating and expanding CTCs both in vitro and in vivo. CTC cell lines representing diverse tumor types may be characterized, authenticated, and collected in a CTC biobank, as is the case for the many PDX biobanks used for preclinical investigations. These CTCs biobanks may prove to be powerful resource for multi-omics and therapeutics research. Considering the current poor

prognosis of metastatic breast cancer, these CTC-derived preclinical models for basic and preclinical research offer great hope for the identification of novel biomarker signatures, therapeutic drug development and testing, and enhancing our understanding of drug resistance in cancer, so that the promise of precision medicine and improved clinical outcomes for patients with metastatic breast cancer may be achieved.

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Circulating Tumor Cells (CTCs) Heterogeneity in Metastatic Breast Cancer: Different Approaches for Different Needs

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Abstract

In metastatic breast cancer the role of circulating tumor cells (CTCs) enumeration for predicting clinical outcome is supported by many studies, most of them dealing with strictly epithelial cells. However, it is becoming clear that CTCs are a heterogeneous cell population characterized by plasticity and including also cells which have lost the epithelial phenotype. Here we review literature data on CTC heterogeneity both at phenotype and at molecular level and discuss the possible contribute of single cell analyses in precision medicine. We conclude with some remarks about the steps still necessary to achieve clinical validity and utility when considering also CTC phenotypic and molecular heterogeneity beyond a simple enumeration.

Keywords

 $\begin{array}{l} Breast \ cancer \cdot \ Metastasis \cdot Circulating \ tumor \\ cells \ (CTCs) \cdot CTC \ heterogeneity \cdot CTC \\ molecular \ characterization \end{array}$

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6.1 Introduction

Metastasis is the leading cause of death in cancer patients, but still the biology of tumor dissemination and metastases formation is poorly understood. Contrary to what initially thought, tumor cell dissemination leading to the formation of clinically overt metastases is a process that starts early [1, 2]. Cells giving origin to metastases improve their fitness for invasion and colonization by acquiring new characteristic either in parallel to the primary tumor or within the primary tumors as progression occurs giving raise to late dissemination. In particular, metastatic cells lose drug sensitivity respect to the primary tumor [3]. Moreover, in metastatic disease, the primary tumor itself is characterized by increasing heterogeneity due to its own evolution and to the reseeding of cancer cells among different sites [4].

Circulating Tumor Cells (CTCs) are the seeds of metastases and their study is instrumental for understanding the metastatic process and tumor complexity. In fact, CTCs originate from established tumor masses, either primary or metastatic foci, migrate into the bloodstream, and acquire the potential to change their fate by undergoing different phenomena driven by epigenetic events, but also by interaction with other cells in the blood such as platelets that enable them to seed metastases [5]. The capacity to lose the lineage commitment for acquiring different features and to direct cell fate by switching to another

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differentiated cell type is defined as "plasticity". CTC plasticity includes different programs affecting invasion, survival and proliferation, and is to a certain extent mirrored by the typical heterogeneity of cancer [6, 7]. Moreover, CTCs reflect in part the spectrum of mutations present in either the primary and/or metastatic tumor [4].

Heterogeneity is a hallmark of cancer that is responsible for its complexity, tracks its development and is regarded as a main culprit for the failure of cancer therapies [3]. CTCs in particular are characterized by different types of heterogeneity: an extrinsic and an intrinsic one. The extrinsic heterogeneity of CTCs is linked to the tumor of origin, and is therefore due to the tumor type and to its specific tumor driver mutations. All these factors give rise to different tumor cell phenotype even within the same tissue. Intrinsic heterogeneity of CTCs instead deals with mechanisms of adaptation occurring during the metastatization process that are instrumental for tumor spread, and includes conversions between cellular phenotypes [6].

The main mechanisms by which CTCs develop intrinsic heterogeneity is the epithelialto-mesenchymal transition (EMT) that is influenced by the type of tumor, the tissue of origin, the local microenvironment of cancer cells and by the treatment. Later, before the colonization step, the disseminated cancer cells undergo an inverted process called mesenchymal-toepithelial transition (MET) that allows their settlement in metastatic foci. The EMT core network controls feedback loops between the two extreme fates (epithelial and mesenchymal phenotype). Those however, are not binary processes, accordingly cells retaining a hybrid epithelial/mesenchymal phenotype are often observed and can promote multicellular aggregate migrations thanks to their mixed traits. Thus, genes regulating the EMT phenotype are differently expressed in distinct CTC populations, promoting their intrinsic heterogeneity and influencing drug resistance and tumor dormancy. Importantly, intermediate states could also induce cells to exhibit stemness traits, although, the acquisition of mesenchymal and stemness traits can be uncoupled [4, 7, 8].

In such a scenario, the key question is how to better understand and possibly best classify CTC heterogeneity in order to obtain a biomarker or a set of biomarkers useful for clinical applications in metastatic patients. It is in fact important to establish which approaches are more suitable for defining CTC features that have a direct impact on prognosis, treatment response prediction and that are able to inform the clinical decision by also taking into account intra-patient heterogeneity, both in space and time.

In this chapter, the CTC heterogeneity issue will be reviewed limiting to Metastatic Breast Cancer (MBC).

6.2 CTC Phenotypic Heterogeneity

CTCs can be isolated from patients' peripheral blood and are used to monitor tumor cell populations during disease progression and in response to therapies. Many CTC isolation technologies have been developed, but only the CellSearch[®] system was warranted FDA-approval. It is based on enrichment of CTCs by epithelial markers (EpCAM) and subsequent software-assisted manual enumeration of CTCs defined as nucleated cells expressing cytokeratins and not expressing the pan-leukocyte antigen CD45 [9].

The role of CTC detection by CellSearch[®] as a survival predictive biomarker in MBC has been extensively tested by Cristofanilli et al. [10]. In patients with measurable metastatic disease, the detection of \geq 5 CTC/7.5 mL of blood before treatment was demonstrated to represent an independent predictor of both progression-free survival (PFS) and overall survival (OS). In a later study, Cristofanilli et al. followed CTCs enumeration in serial samples collected at different times, showing that their detection before firstline therapy and after 4 weeks of treatment was significantly predictive of PFS and OS. Persistence of CTCs at restaging time was also significantly associated with worst prognosis [11].

In a pooled analysis including 51 centers across Europe, Bidard et al. reported the clinical

validity of CTCs changes during treatment and showed that addition of CTC enumeration to prognostic model including currently used clinicopathological variables improves prognosticaadding significant independent tion by information [12]. In keeping with this, in a recent retrospective study pooled analysis including almost 2500 MBC patients, CTC enumeration proved to be able to stratify patients based on their disease aggressiveness (indolent vs aggressive) supporting the introduction of CTC to improve staging in MBC [13].

To improve CTC enumeration by the CellSearch® system, de Wit et al. studied the relevance of EpCAM^{low}-expressing cells, which are considered as EpCAM-negative cells by the CellSearch® system and discarded. EpCAM^{low}-CTCs were collected on microfilters from the blood fraction discarded after CellSearch® CTCenrichment, were fluorescently labeled and scored for enumeration using the classic CellSearch® critera. The authors analyzed the presence of these cells in the blood of castrationresistant prostate cancer and MBC patients. In both tumor types the number of patients with positive detection of CTCs increased when both EpCAM^{high} and EpCAM^{low} CTCs were considered. However, the presence of EpCAMlow CTCs was not associated with survival, and it deserves to be further investigated [14]. These cells may in fact be a different subpopulation of CTCs, however, although their epithelial origin is proven by the expression of cytokeratin (CK), no direct evidence exist for their malignant nature. Only genetic analysis could provide direct evidence and help clarifying specific features of such cells.

Despite the success of CellSearch[®] in predicting risk in MBC, concerns arise on the possible clinical role of CTC subpopulations that do not strictly meet CellSearch[®] criteria and that are missed by such method. Therefore, studies aiming at selecting and identifying all CTC subpopulations have been done using marker-independent approaches for CTC-enrichment such as magnetic beads selection (AdnaTest, Myilteni Biotec), filters (ScreenCell), gradient centrifugation (Oncoquick[®]), size and deformability selection (ParsortixTM) and exploiting dielectrophoretic properties (DEPArrayTM System) [15, 16].

The DETECT study run a direct head-to-head comparison between CellSearch® and the AdnaTest on a prospective series of 254 women with MBC. Fifty percent of patients were defined as CTC-positive by the CellSearch® system versus 40% by the AdnaTest, which employs magnetic beads functionalized with antibodies against EpCAM and MUC1 for CTC-enrichment and a multiplex PCR assessing EPCAM, MUC1 and HER2 expression for CTC detection. Overall the concordance rate between the two assays was 64%, but no data on the direction of discordances were reported. The association between CTC positivity by CellSearch[®] and shorter PFS and OS was confirmed, whereas no statistically significant associations were observed between AdnaTest CTC-positivity and clinical outcome [17].

To better investigate the impact of CTC detection methods, also Aaltonen et al. studied CTCs from MBC patients in parallel by CellSearch[®] and AdnaTest. They used a new developed kit for CTC capture, the EMT2, which adds antibodies against HER2 and EGFR to the CTC-enrichment antibody cocktail to improve the capture efficiency compared to the traditional AdnaTest (EMT1). Enriched samples from AdnaTest EMT1 and EMT2 were then analyzed by multiplex qPCR for 38 genes associated with cancer. Evaluating CTC-positivity, a number of patients was positive by both methods, whereas some patients resulted as positive only by CellSearch® or AdnaTest. In addition, some of the samples defined as CTC-negative by both the CellSearch® and the classic AdnaTest (based on transcripts for EPCAM, HER2, MUC1), did instead express KRT19 or ERBB2 questioning their negativity [18]. These results, although lacking strong clinical evidence, definitely highlight the potential of combining different markers to improve circulating cells classification and possibly their association with clinical outcome.

Using the AdnaTest only, Aktas et al. investigated expression of EMT markers (Akt-2, Twist1 and PI3K α) and of ALDH1 (marker for stem cell) in different MBC patients undergoing different types of palliative therapy. Treatment response was evaluated according to RECIST criteria. Only 10% of responders were CTC-positive, respect to 71% among non-responder group. Interestingly, in 81% of CTC-positive patients, at least one of EMT markers, ALDH1 or both, were expressed. Conversely EMT and stem-cell markers were expressed in only 11% of CTC-negative samples. Such data suggest that beside the presence of CTCs themselves, also their specific transcriptomic program needs to be considered in order identify CTC subpopulations and understand clinical associations [19].

Overall, the conclusions of the above described studies lead us to the exploration of circulating cells exhibiting mesenchymal traits.

The presence of mesenchymal and intermediate epithelial/mesenchymal cells in blood samples enriched for CTCs in MBC patients was investigated in a landmark study by Yu et al. [20]. These authors investigated the presence of cells exhibiting mesenchymal traits both in primary tumor and in blood samples and reported only rare mixed epithelial/mesenchymal cells within the primary tumors whereas mixed phenotype cells were frequently present among CTCs. Blood samples were enriched for CTC with the microfluidic herringbone-chip using epithelial and tumor-specific antibodies (EpCAM, EGFR, HER2) and studied at single cell level with in situ approaches. Based on the results of RNA-ISH analysis that evaluated a series of epithelial (KRT5, 7, 8, 18, 19; EpCAM, CDH1) and mesenchymal (FN1, CDH2, SERPINE/PAI1) markers five categories of CTCs were defined: exclusively epithelial cells (E), 3 categories of intermediate cells (E > M, E = M, E < M), and exclusively mesenchymal cells (M). Using a cutoff of 5 CTCs/3 ml 41% of MBC, at various treatment stages, scored positive for CTCs, and EMT features were different between lobular and ductal histotypes. Also when comparing pre- and post-treatment blood samples (n = 10), different CTCs features were found. In post-treatment samples from patients who responded to therapy (n = 5), the absolute CTC numbers decreased and/or the proportion of M-CTCs decreased. Conversely, in patients who experienced progression while on therapy (n = 5), the number of M-CTCs increased in the post-treatment samples supporting a role of EMT in treatment sensitivity [20].

To understand the prognostic relevance of single CTCs with specific phenotypes, Papadaki et al. detected and characterized CTCs pre- and post-treatment in patients with MBC. They identified four different CTCs subpopulations by performing triple immunofluorescence on cytospin preparations of peripheral blood mononuclear cell (PBMC) with antibodies against CK8, CK18 and CK19 (epithelial markers), ALDH1 and TWIST1. In patients not responding to the treatment, the number of samples with CTCs showing stem and partial EMT features increased. Conversely, positivity percentages slightly decreased after treatment for the other types of CTCs (*i.e.*, CTC showing stem but lacking partial EMT features, and CTCs lacking stem features and/or positive or negative for EMT features). In keeping with this, only the presence of CTCs with stem and partial EMT features was associated to shorter PFS and OS. This finding was interpreted by the authors as a suggestion that partial EMT increases the chance of the cells to subsequently undergo mesenchymal-epithelial transition (MET), a step necessary to allow colonization at the metastatic site [21].

The importance of such mixed-phenotype CTCs is also supported by other studies. Using CD45 MicroBeads for depletion of leukocytes and the DEPArrayTM system, Bulfoni et al. identified four different CTC subtypes: epithelial CTCs (E-CTC) expressing only epithelial markers (EpCAM, E-cadherin), CTCs undergoing EMT (EM CTC) co-expressing epithelial and (CD44, mesenchymal markers CD146, N-cadherin), putative mesenchymal cells (MES) expressing only mesenchymal markers, and negative cells (NEG) not expressing the tested markers. Some associations were highlighted between CTC subpopulations and breast cancer molecular subtype, proliferative rates and metastatic localization, however only the EM-CTCs were significantly associated with shorter PFS and OS [22].

The identification of the so far described CTCsubpopulations is strongly influenced by the type of CTC-enrichment. In such a context, to avoid underestimation of CTC subpopulations, the ParsortixTM system that selects CTCs in an epitope-independent way, exploiting size and deformability as selection criteria appears to be particularly promising [23]. In our laboratory we combined the ParsortixTM with the DEPArrayTM system. Thanks to the presence of a fluorescent microscope equipped with a camera, and to a microfluidic chip exploiting dielectrophoresis to entrap single cells, the DEPAarrayTM allows visualization of cells labeled for epithelial, leukocyte, mesenchymal or other type of markers, coupled with the selection and recovery by the operator of the cells of interest [24]. Using this system, we were able to observe the presence of specific CTCs subpopulations in all blood samples (n = 14) from women who underwent mastectomy for early triple negative breast cancer (TNBC), collected at the time of imaging-proven distant site relapse. Our data support the concept that CTCs identification cannot rely on a single CTC phenotype, but should rather broaden the phenotypic criteria used for selection and include direct molecular evidence for the malignant nature of the selected cells. In fact, using antibodies against epithelial (EpCAM, panCK, EGFR) and leukocyte (CD45, CD14, CD16) markers, we succeeded in distinguishing two different CTCs subpopulations: epithelial CTCs (eCTCs), and non-conventional "putative" CTCs (ncCTCs), i.e. cells lacking both leukocyte and epithelial (tumoral) but with malignant genotype. We therefore suggest that besides mixed CTC (epithelial and mesenchymal phenotype) a third CTC subpopulation characterized by the lack of expression of epithelial and leukocyte markers, but with confirmed aberrant genotypes is detectable in the blood collected at the time of imagingdocumented relapse, of women who underwent mastectomy for early TNBC (manuscript under preparation).

Whereas many studies have addressed both technical aspects involved in isolation of CTC subpopulations and the clinical role of CTC subpopulations, few studies are instead available on the mechanisms involved in induction of mesenchymal traits in CTCs. Interaction with platelets and secretion of TGF β have been described as a possible mechanism for induction of EMT [5] and some heterogeneity in methylation of genes involved in EMT has been reported for single CTCs [25] thus suggesting an epigenetic control.

Recently a different mechanism has been suggested as possibly causally involved in promoting CTC heterogeneity and in the generation of specific CTC subpopulations, i.e. heterotypic cell fusion between epithelial cells and blood cells, in particular with macrophages [26]. Such a mechanism, so far experimentally investigated in preclinical models but poorly validated in clinical samples, deserves further attention as it may open the way to the identification of even more CTC subpopulations and possibly also to new pharmaceutical targets for interfering with tumor dissemination.

All the above reported data emphasize that studies evaluating the role of circulating cells in cancer evolution should not be limited to circulating cells expressing epithelial and lacking leukocyte markers, but must be broadened to include other phenotypes. Unfortunately, at the moment clinical data available on CTC subpopulations are not impressive and often limited to small studies lacking statistical power: nonetheless the field appears as very promising. However, we must underline the lack of both clearly defined criteria for selection and of a proof of the malignant nature of the various CTC subpopulations.

6.3 CTC Molecular Characterization

Discrepancies between different methods for CTC-identification by phenotypic features suggest the need for more accurate criteria for CTCs classification. In fact, as described above the phenotype alone is not sufficient to classify a single cell as a *bona-fide* CTC and thus in the case of ncCTCs (i.e. CTCs lacking the conventional identification markers) only a characterization of the genotype can definitely ascertain the actual malignant nature.

A possible approach to test for the malignant nature is performing an analysis of copy number alterations (CNA) at a single cell level. By running a low-coverage whole genome sequencing it is in fact possible to obtain CNA profiles for each cell, indicating if the genome is diploid and thus presents a flat CNA profile as expected for normal cells or if, as expected for a *bona-fide* CTC, it contains regions characterized by genomic gains and losses. This approach, which leads to a molecular proof of tumor origin for each singlecell, is particularly important for cells where the phenotype does not allow a clear distinction.

Molecular characterization however, does offer much more than simply ascertaining the malignant nature, as it contains the information on the clonal origin of each analyzed cell.

In current practice tissue biopsies are used to test tumors for actionable genomic abnormalities despite well-known limitations dealing with spatial and temporal heterogeneity. In the metastatic setting, limitations affecting tissue profiling are even greater, since the tumor cells are homed in different anatomical sites and might have evolved in distinct ways. In this context, the analysis of CTCs deriving from both primary tumor and metastatic lesions would instead provide a comprehensive molecular portrait of the entire tumor burden just by a single blood test. In principle, obtaining a molecular profile of CTCs could therefore facilitate individual patient treatment management thus helping to reach the ambitious aim of achieving a true precision medicine. However, although this approach appears promising from a theoretical point of view, it still has many limitations. Accordingly, revision of the literature data mostly shows results obtained on very few patients and by different technical approaches that limit comparability. Still some general messages can be derived.

Among the first in assessing the feasibility of mutational analysis on single CTCs isolated with the DEPArrayTM, Mu et al. investigated mutations in CTCs isolated from one woman with inflammatory MBC. CTCs were enriched by an unbiased method based on size selection, using ScreenCell filters, and were thereafter analyzed with the DEPArrayTM to select and isolate single

CTCs. After whole genome amplification (WGA) using Ampli1TM WGA kit, mutational analysis on amplified DNA from 7 CTCs was performed by Sanger sequencing, in order to investigate the presence of a specific TP53 mutation which had been previously identified in the primary tumor: the TP53 exon 6 p.R248W missense point mutation. The same mutation was investigated both in single and pooled CTCs. Heterozygous TP53 mutation was found in 1 single cell and 1 pool of 3 CTCs, and homozygous TP53 mutation was instead detected in 1 single CTCs and 1 pool of 2 CTCs. The mutation was not present in one WBC analyzed as control [27]. These results show the feasibility of a molecular approach to investigate relevant mutations in CTCs, and suggest that mutations identified in the tissue can also be traced in single or pooled CTCs, which might therefore be regarded as representative of the tissue of origin. No information is however provided on the possible presence of CTC-private mutations that may have resulted from tumor clonal evolution, as has instead been reported in other clinical settings [28].

CTC private mutations, if identified with reliable methods, are very interesting since they provide a genomic/clonal tracking of the disease evolution. In the case of MBC, searching for CTC private mutations in the ESR1 is particularly promising, since those mutations (some of them directly involved in endocrine treatment resistance) are rarely detected prior to treatment start and only appear with the onset of treatment resistance [29, 30]. This represents therefore a typical clinical scenario where CTCs molecular analysis would be useful to dissect time-related heterogeneity. Moreover, CTCs represent the ideal biological sample for tracking in real-time the onset of endocrine resistance, since they potentially allow evaluating at the same time both mutations as well as splicing variants. Nonetheless, a recent study planned to evaluate ESR1 mutations and splice variants in CellSearch-enriched bulk CTCs before start of endocrine therapy and at the time of progression, failed to detect an enrichment for *ESR1* mutations at progression [31]. Such mutations were instead found to be enriched in ctDNA. These results, which apparently rule out a role for CTCs in monitoring the onset of endocrine resistance, as suggested by the authors themselves, can be instead interpreted as an indication of the need to perform CTC molecular analyses on single isolated cells rather than on bulk samples, albeit enriched for CTCs. Indeed, only by considering the genotype of each single CTC we may be able to capture their message on heterogeneity and evolution of the disease. This poses technical problems that can be overcome as reported above, but also challenges in the interpretation of results.

In a study to assess the possibility of detecting mutations in CTCs, Paolillo et al. investigated ESR1 mutations by Sanger sequencing in single CTCs from MBC patients. They analyzed 40 CTCs recovered combining CellSearch® and DEPArray[™] platforms from 3 ER-positive MBC patients. Their protocol was technically robust since 12 white blood cells (WBCs) analyzed as controls were all correctly classified as wild-type for ESR1. The first investigated patient presented 5 CTCs, all wild-type for ESR1. The second patient carried a single *ESR1* activating mutation (Y537S) in exon 8 in heterozygosis in 3 CTCs, and the same mutation in homozygosis in 1 CTC, the last CTC was instead wild-type for the same mutation. In this patient, CTCs' molecular heterogeneity and the detection of activating mutations were in keeping with the observed treatment failure. For the third patient, serial samples collected during treatment at different time points were available. In the first sample, the authors could study 12 CTCs, all wild-type for ESR1 mutations, thus suggesting that the patient was still endocrine sensitive. In the second blood sample, the authors detected high heterogeneity: 8 CTCs were negative for estrogen receptor (ER) expression and wild-type for ESR1 mutation, 4 CTCs were positive for ER expression and wildtype for ESR1 mutation, 3 CTCs were positive for ER expression and carried the mutation Y537s in heterozygosis, and 1 CTCs was positive for ER expression and carried 2 different mutation in exon 8 of ESR1 in homozygosis [32]. In this latter patient too, the appearance of ESR1 mutations in CTCs was mirrored by failure to respond to conventional endocrine treatment. Overall, these results, although still anecdotal, show that CTC characterization might be more informative than tissue, giving new hints on resistance mechanisms to endocrine therapy in ER-positive MBC patients.

In the clinical management of MBC the most frequently used treatment-predictive biomarkers, such as ER and HER2, are evaluated at protein level on the primary tumor. Thus, besides molecular characterization at genomic level, also transcriptomic analysis of single CTC should be useful to guide treatment and to inform on possible changes of the molecular phenotype with respect to the primary tumor. Such studies would indeed provide hints on the heterogeneity of typical treatment targets.

In a recent study, the status of the therapeutic biomarkers ER and HER2 was examined in **CTCs** isolated from 105 women with MBC. Immune enrichments for EpCAM and FACS analysis were used for isolation of single cells prior to performing genome wide CNA by aCGH and transcription analysis of 64 genes by low-density array qPCR. Combined transcriptional and genomic profiling showed presence of different CTCs subpopulations at different frequencies (26% ESR1 - ERBB2-, 47% ESR1 + ERBB2-, and 27% ERBB2+). Serial testing of longitudinally collected samples showed that ERBB2 status was more stable over time compared to ESR1 status. Moreover, discordance in ESR1/ER (27%) and ERBB2/HER2 (23%) status between CTCs and matched primary tumors emerged by comparative analysis [33]. Based on the results it was concluded that CTC molecular analysis has the potential to help treatment decision in a clinical setting of a progressing disease, but the clinical utility of CTCbiomarkers is far from being demonstrated.

All studies listed above are concordant about the concept that CTC molecular analysis could improve clinical practice, although so far they only demonstrate the technical feasibility. The reported results underline a high level of intrapatient heterogeneity indirectly suggesting that currently used criteria for patients' stratification could in some cases not represent the patients' real tumor "status". Patients classified as similar at tissue level could instead display differences at molecular level, therefore molecular approaches represent an opportunity for more accurate patients' profiling for personalized medicine.

In the meantime a new scenario is slowly appearing, where liquid biopsy can improve understanding of metastatization processes bringing out different characteristics between the primary tumor and the circulating tumor elements and elucidating the extrinsic heterogeneity of the tumor. Single-cell analysis may also help to rebuild the origin of detected variants, understanding if they coexist in a single cell or derive from distinct clones.

Using the CellSearch® system for CTC enrichment, the DEPArrayTM for single cell isolation and next generation sequencing (NGS), De Luca et al. investigated mutations in single CTCs in patient with MBC. In this study, the authors could compare CTCs and primary tissue (limiting to the variants found in the single CTCs) in 3 patients, and they found correspondence only for a benign PDGFRA variant (1 patient) and a deleterious somatic mutation in TP53. For all the other variants discovered in CTCs, there was no correspondence in primary tissue [34]. After analyzing 14 CTCs derived from 4 patients, for 51 sequence variants in 25 genes, it was observed that almost all mutations were present in only one single CTC. This represents an interesting result, which might highlight the importance of single cell analyses for studying heterogeneity, but it also poses technical questions. The mandatory step in single-cell analyses of performing a WGA might have been responsible for the introduction of technical artifacts and, despite the fact that high coverage increases the confidence of variants called in each single cell, DNA polymerase fidelity still represents a concern. However, also given the technical reliability of the data, can such result be considered robust enough for a clinical decision? This is at the moment the main issue, questioning the application of single cell NGS in the clinics. Nonetheless, this is also possibly its strength, due to the ability to potentially suggest new biologically/clinically relevant variants as it is illustrated by the example reported below.

Paoletti et al. investigated the possibility of obtaining paired information from CTCs and tissue metastases by NGS in 12 patients. For this purpose the authors processed whole blood from MBC patients using the CellSearch® system, isolated single cells with the DEPArrayTM system and applied Ampli1TM protocol for DNA isolation and analysis. Targeted NGS was performed using the Oncomine Comprehensive Assay, and selected mutations were confirmed by Sanger Sequencing. In parallel, frozen tissue from primary tumor and fresh tissue from metastatic biopsies were used to generate exome libraries to be processed for Whole Exome Sequencing (WES). High concordance (85%) between CTCs and fresh metastatic tissue was found, in term of genomic alterations. However, private alterations were detected both in tissue and in CTCs, though at low frequencies [35]. Since potentially clinically informative/actionable mutations may either be exclusively present in metastatic tissue or in CTCs, the authors suggest performing genomic profiling of both, since results may be considered as complementary in order to achieve a true clinical impact.

Within the same study, in one single patient with endocrine treatment refractory disease, 32 individual CTCs and CTC-pool samples were recovered and analyzed by comprehensive NGS revealing the presence of a well-known ESR1 mutation associated with endocrine resistance, the ESR1p.Y537S. However, in one single CTC such mutation was not detected, and a new heterozygous ESR1 mutation (ESR1p.A569S) was instead detected and confirmed by ddPCR (droplet digital PCR). To assess its role, Paoletti et al. stably overexpressed the ESR1p.A569S mutation by lentivirus-mediated infection in the endocrine sensitive MCF7 cells showing increased estradiol and tamoxifen-induced growth, thus validating its role in conferring a modest treatment resistance. In this case, a specific somatic variant detected in a single CTC allowed increasing our knowledge on endocrine resistance mechanisms.



Fig. 6.1 Schematic representation of the steps in the validation of CTC as a biomarker. Vertical bars represent the consecutive steps in the path towards the development of a biomarker: technical validity, clinical validity and clinical utility. Arrows represent the current achievements and

With the exception of specific cases as the one reported by Paoletti et al. detection of a somatic mutations in one single CTC is still of questionable biological or clinical utility. In a study run on 112 women with MBC, CTCs were enumerated by CellSearch[®], and 5 patients with CTC counts ≥ 100 were chosen for performing targeted NGS (Custom Cancer Hot Spot panel V2), globally analyzing 40 CTCs. Mutational heterogeneity was observed among CTCs, and it was reflected by ctDNA analysis run in matched samples. Minor subclonal mutations, likely acquired during tumor progression, were observed only in liquid biopsy and were undetectable at tissue level. These data suggest that ctDNA mutational profile can reflect the CTC heterogeneity in patients with high CTC counts, however no data are provided on patients with low CTC counts. It may be concluded that, despite the good correlation with ctDNA, still the occurrence of a new somatic variant in one single CTC remains difficult to interpret [36].

dots represent future steps necessary for considering CTC-enumeration, CTC-phenotypic characterization, CTC-molecular characterization and CTC-functional characterization as a biomarker

6.4 Conclusions and Future Perspectives

Since the original observation of the presence of tumoral cell in the blood of patients with solid tumors, enormous progresses have been made. In last two decades, thanks to efforts in technical standardization, enumeration of epithelial-CTCs has become a widely used tool for prognostication and treatment monitoring across many tumor types. Indeed, in MBC CTC enumeration with the CellSearch[®] is technically and clinically valid [12] and has been proposed as useful for clinical staging of MBC [13].

Now, thanks to new technical refinements both in CTC enrichment and in CTC molecular characterization, the possibility of using CTCs for a real-time assessment of the disease and of its evolution at molecular level is becoming reality. However, there are still many aspects that need to be better investigated. Among those, although we know that CTC are present as phenotypically distinct subpopulations, and we have achieved at least a technical validation of the methods used for detection of CTC subpopulations, we still ignore the role of each subpopulation and are thus far from having obtained a clinical validation for each CTC phenotype.

In addition, molecular characterization of single CTCs is technically feasible both at genotype and at transcriptional level. So far results are definitely suggestive of a possible role of CTC as tissue surrogate, and as a means to capture and to overcome tumor heterogeneity, but again we are far from having reached a clinical validation and still confused about the possible future clinical utility. We are however confident on the fact that further advances in methods that not only facilitate CTC isolation and characterization, but also allow their functional characterization, will bring us closer to achieve clinical utility (Fig. 6.1).

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Abstract

Metastasis is the major cause of mortality in patients with breast cancer; however, the mechanisms of tumor cell dissemination and metastasis formation are not well established vet. The study of circulating tumour cells (CTCs), the metastatic precursors of distant disease, may help in this search. CTCs can be found in the blood of cancer patients as single cells or as tumor cell aggregates, known as CTC clusters. CTC clusters have differential biological features such as an enhanced survival and metastatic potential, and they hold great promises for the evaluation of prognosis, diagnosis and therapy of the metastatic cancer. The analysis of CTC clusters offers new insights into the mechanism of metastasis and can guide towards the development of new

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diagnostic and therapeutic strategies to suppress cancer metastasis. This has become possible thanks to the development of improved technologies for detection of CTCs and CTC clusters. However, more efficient methods are needed in order to address important questions regarding the metastatic potential of CTC and future clinical applications. In this chapter, we explore the current knowledge on the role of CTC clusters in breast cancer metastasis, their origin, metastatic advantages and clinical importance.

Keywords

Breast cancer · Circulating tumor cells (CTCs) · CTC clusters · Metastatic potential · Homotypic CTC clusters · Heterotypic CTC clusters

7.1 Introduction

Breast cancer (BC) is the most common cancer in women worldwide. It exceeded 2 million new cases diagnosed in 2018, representing about 25% of all cancers in women [1]. Despite advances in prevention, diagnosis and treatment, about 5–10% of patients show metastasis at the time of diagnosis and near a 30% will develop metastasis throughout the course of treatment [2]. The

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metastatic stage remains an incurable malignant disease, accounting for more than 625,000 deaths per year worldwide (WHO). The ultimate responsible of seeding cancer metastasis are the circulating tumor cells (CTCs), which hematogenous spread was reported as early as in the nineteenth century. CTCs are found in the blood of cancer patients as single cells, although small groups of these cells named CTC clusters have also been detected, and very importantly, their presence is associated with an earlier onset of metastatic disease [3–6].

The advancement on the development of technological platforms able to isolate and to identify individual CTCs and CTC clusters from blood samples has been key to overcome the difficulties of working with a population of rare cells, extremely infrequent in the case of CTC clusters. It is estimated that only a 3,4% of CTCs are clusters, and that about 50% of patients with metastatic breast cancer (MBC) have at least one cluster [4]. For this reason, most of the knowledge gained about the contribution of CTC clusters to metastasis and their clinical implications has been achieved in the last decade, and it suggests that CTC clusters may represent one of the key mechanisms initiating the metastasis process. However, the biological features of CTC clusters such as genesis and the underlying molecular mechanisms of their metastatic competency, remain largely unknown.

In this chapter, we will discuss the role of CTC clusters in breast cancer metastasis, focusing on their biological features and clinical implications.

7.2 Insights on the Existence of CTC Clusters

The first experimental evidences of the presence of tumor cells in blood circulation were made by Langenbeck's in 1841 [7], followed by the observations in 1869 by the Australian physician and pathologist Thomas Ashword, who also reported the presence of tumor cells in the blood of a male patient with metastatic cancer [8]. The vast majority of CTCs in circulation are found as single cells, and just a small proportion are represented by CTC clusters. By definition, a cluster of CTCs is a group of more than two tumor cells detected in the blood of a cancer patient, moreover the size of the clusters can vary from 2 tumor cells up to >100 cells [5]. Different names have been used in the literature to describe aggregates of tumor cell. CTC clusters have also been referred as circulating tumor microemboli (CTM), circulating micrometastases, circulating tumor aggregates, and tumor cell clumps [4, 5, 9,10]. For an easy understanding on the development of this chapter, we will refer to them as CTC clusters.

The existence of clusters of circulating tumor cells was already predicted by Rudolf Virchow, in 1858. In his theory about the dissemination of tumor cells, he hypothesized that metastasis could be explained simply by the arrest of tumorcell emboli in the vasculature [11]. It was almost a century later that some initial studies emerged acknowledging the role of CTC clusters in metastasis, although at the time the term emboli, and not cells, was widely used to describe these tumor aggregates [12, 13]. In 1954, the pioneering work by Watanabe showed that clumps of viable carcinoma cells injected intravenously in mice were able to form lung metastases much more efficiently than single cells suspensions, when injected at equal numbers [14]. Watanabe showed that the total number of cells injected was, apparently, not an important factor and he suggested that clumps of cells have a survival advantage as compared to single cells. This data represents the initial indication of the greater predisposition of CTC clusters to form distal metastasis than single CTCs. Short after, in the 1970s, similar results were obtained in preclinical studies employing metastatic models to lung of melanoma, fibrosarcoma, and mammary tumor cells, corroborating the highest efficiency of CTC clusters to form distant metastasis [10, 15–17]. These studies also showed that the success rate of CTC clusters on the formation of metastases partially depends on

the size and the concentration of the clusters found in the blood. Moreover, they revealed that CTC clusters are able of passaging through the circulation vessel in the lungs of experimental animals [17–20]. Similarly to these preclinical studies, the entrapment of CTC clusters in the microvasculature of patients has also been reflected more recently in the literature. Commonly, these tumor cell aggregates are found during autopsy of cancer patients [21, 22]. Thus, lungs might retain a substantial number of CTCs, and clusters of tumor cells (named tumor cell emboli) were observed in three out of eight patients with MBC [22]. Furthermore, a postmortem analysis of a patient with metastatic Triple Negative breast cancer showed the presence of tumor emboli in diverse metastatic locations such as brain and lungs [21].

Despite of these initial findings, the advance on the understanding on the behavior and biology of CTC clusters and the appreciation of their full contribution to the process of metastasis was hampered mainly for one reason, the lack of technology available with the sufficient sensitivity to detect small populations of CTCs, including CTC clusters, and able to distinguish them from blood cells. Even nowadays, a key challenge is to develop enrichment technologies capable of capturing intact CTC clusters avoiding breaking them apart [23]. In this regard, it is worth reminding that if the presence of CTCs in the blood of cancer patients is rare, the presence of CTC clusters is extremely rare [24], representing only 2-5% of all CTCs, according to clinical and preclinical studies [4, 6].

On the other hand, the established view of the metastatic process as described by the clonal evolution model by Peter Nowell in 1976 [25], by which metastatic tumors arise from the proliferation of individual CTCs disseminated into distant organs [26, 27], has also contributed to the slow advancement on the study of CTC clusters.

Although the existence of CTC clusters has also been known for decades, it was in the 1990's that the first studies isolated CTC clusters from the blood of patients with prostate, colorectal, breast, lung cancer and clear cell renal cell carcinoma [3, 5, 28–31]. A summary of the studies in which CTC clusters have been investigated in the blood of patients from different cancer types is shown in Table 7.1. Since then, recent technological advances, developed mainly in the last two decades, have enabled a more efficient isolation of CTCs and CTC clusters from the blood of cancer patients and thus, the significance of CTC clusters has emerged as a functional entity in the metastatic process (Fig. 7.1).

7.3 Challenging the Traditional View of the Dissemination Process

The traditional view on the development of metastasis believed that the establishment of metastatic tumors is due to the proliferation of individual CTCs released by the primary tumor into distant organs [26, 27]. Within this scenario, if the "seed" of the metastasis is a single CTC, then the resulting tumor will be clonal. However, this conventional model of cancer metastasis has been challenged by data extracted from recent genomic studies tracking the evolutionary histories of tumor cell clones along the metastasis progression, which show that metastases can be composed of multiple genetically distinct clones. Thus, in murine models of breast cancer, pancreas and small cell carcinoma [6, 45, 66], and in patients with metastatic prostate cancer [67], the presence in high frequency of polyclonal metastases has been observed. Along with these evidences, the isolation from the blood of cancer patients of CTC clusters and their capacity to seed distant metastasis (later discussed) suggest that, if a CTC cluster is the "seed", the resulting metastasis can be polyclonal. These observations together with some other experimental evidences suggest that different clones of tumor cells can show cooperabehavior, a concept called "clonal tive cooperation", promoting their mutual survival and metastatic capacity [68–70]. Therefore, it is feasible to speculate that CTC clusters can be formed by the combination of different clones harboring diverse biological properties regarding survival and growth.

		Method for CTC cluster	
Tumor type	Evaluation	enrichment	References
Colorectal cancer $(n = 32)$	Detection	Immunomagnetic cell	[28]
		separation	
Liver cancer $(n = 44)$	Detection and prognostic value	ISET®	[32]
Prostate cancer $(n = 15)$	Detection	HBCTC-Chip	[33]
Non small-cell lung cancer stage III–IV (n = 28)	Detection	ScreenCell [®] Cyto filter	[34]
Lung cancer $(n = 6)$	Detection, EMT features and apoptosis	ISET [®] and CellSearch [®]	[30]
Breast (n = 4), non-small cell lung (n = 14), pancreatic (n = 18), prostate (n = 15) cancer	Detection	High throughput microscopy for immunofluorescence	[4]
Small-cell lung cancer $(n = 97)$	Prognostic value	CellSearch®	[5]
Small-cell lung cancer $(n = 40)$	Comparative detection	CellSearch [®] , ISET [®]	[35]
Pancreatic cancer $(n = 54)$	Detection and molecular characterization	ISET®	[36]
Non-small cell lung cancer (n = 78)	Detection and prognostic value	No enrichment done. Blood cytospin into microscope slide after RBC lysis	[37]
Breast cancer various stages $(n = 41)$	EMT features	HBCTC-Chip	[38]
Non-small cell lung cancer ($n = 22$) and small cell lung cancer ($n = 21$)	Detection and technology comparison	Microcavity array (MCA) system	[39]
Metastatic breast $(n = 5)$ and non-small cell lung $(n = 5)$ cancers	Detection and technology testing	Spiral microfluidic device	[40]
Breast cancer $(n = 79)$ and prostate cancer $(n = 64)$; breast cancer mouse model	Prognostic value, metastatic potential, polyclonal metastases seeding and molecular characterization	^{HB} CTC-Chip	[6]
Breast cancer stage III–IV $(n = 5)$, non-small cell lung $(n = 13)$, and colorectal cancer stage IV $(n = 3)$ patients.	Detection	Flexible micro spring array (FMSA)	[41]
Breast cancer stage III–IV (n = 115)	Prognostic value	CellSearch®	[42]
Triple-negative breast cancer $(n = 60)$	Prognostic value	CellSearch®	[43]
Breast cancer (n = 27), melanoma (n = 20), and prostate cancer (n = 13)	Detection and technology testing	Cluster-Chip	[44]
Pancreatic cancer mouse model	Metastatic potential and polyclonal metastases seeding	No enrichment done. Whole blood was analyzed under the microscope	[45]
Breast cancer mouse model	Metastatic potential and polyclonal metastases seeding	No enrichment done. Blood cytospin into microscope slide after RBC lysis	[46]
Metastatic melanoma (n = 128)	Prognostic value	ISET®	[47]
Colorectal cancer stage IV $(n = 54)$	Detection and correlation with disease progression	CMx [®] platform	[48]
Head & neck cancers early to late stages $(n = 24)$	Detection	Spiral microfluidic device	[49]

 Table 7.1
 Studies evaluating CTC clusters in different cancers

(continued)

		Method for CTC cluster	
Tumor type	Evaluation	enrichment	References
Metastatic breast cancer $(n = 128)$	Prognostic value	CellSearch®	[50]
Metastatic breast cancer $(n = 52)$	Prognostic value	CellSearch®	[51]
Lung cancer $(n = 32)$	Detection	EpCAM-based microfluidic chip	[52]
Pancreatic ductal adenocarcinoma (n = 63)	Prognostic value	CMx [®] platform	[53]
Advanced colorectal cancer (n = 98)	Prognostic value	ISET®	[54]
Gastric cancer stage IV $(n = 41)$	Prognostic value	ISET [®]	[55]
Epithelial ovarian cancer $(n = 54)$	Prognostic value	Biotin-doped Ppy-deposited microfluidic chip	[56]
Head and neck cancer $(n = 53)$	Prognostic value	ISET [®]	[57]
Sarcoma (n = 36)	Detection	CellSieve TM	[58]
Lung cancer early stage $(n = 36)$	Prognostic value	OncoBean Chip	[59]
Pancreatic cancer $(n = 40)$	Prognostic value	NE-iFISH	[60]
Breast cancer $(n = 43)$	Methylation status of CTC clusters	Parsortix TM	[61]
Glioblastoma (n = 13)	Detection and molecular characterization	Parsortix TM	[62]
Breast cancer (n = 118)	Prognostic value, metastatic potential, polyclonal metastases seeding and molecular characterization	CellSearch®	[63]
Head and neck cancer stage I–IV (n = 21)	Detection	Multi-flow microfluidic (MFM) system	[64]
Gastric cancer non metastatic $(n = 55)$	Prognostic value	ISET®	[65]

Table 7.1 (continued)

CMx cells captured in maximum, *EMT* Epithelial to mesenchymal transition, *EpCAM* Epithelial cell adhesion molecule, *ISET* Isolation by SizE of Tumor cells, *NE-iFISH* Negative Enrichment Immunofluorescence and an In Situ Hybridization System, *RBC* red blood cells

In this sense, the mouse as a preclinical model, combined with the performance of lineage tracing experiments, has been a valuable tool to probe the seeding of polyclonal metastases by CTC clusters, in particular in breast cancer. By establishing primary tumors using color coded tumor cells, expressing fluorescent proteins of diverse colors, four independent groups have tested whether metastases arise by accumulation of single CTCs or by the direct seeding of CTC clusters [6, 45, 46, 63]. In the experimental setup involving lineage tracing and tumor transplantation, single colored metastases will arise either from seeding of single CTC and clusters composed by only one color (monoclonal). On the other hand, multicolored metastases will be the result of seeding by CTC clusters composed of more than one color (polyclonal). Three of these

mouse experiments have been conducted in breast cancer models, including patient-derived xenografts (PDXs) models, and the fourth one in a pancreatic cancer model. As a result of these experiments, all groups have found evidences of multicolored metastases, indicating that CTC clusters can seed polyclonal metastases [6, 45, 46, 63]. However, as it will be discussed in the next section, the mechanism by which tumor cells give rise to CTC clusters seems to differ in some cases. The experimental design of these studies however, did not address whether a cooperative behavior was happening between clones. In addition, a similar experiment developed, in which two melanoma cell lines with different metastatic potential were mixed and injected into the flank of nude mice, has shown the presence of polyclonal CTC clusters and polyclonal metasta-


Fig. 7.1 CTC clusters isolated from the blood of metastatic breast cancer patients. Representative images of CTC clusters captured by the epitope-dependent system

CellSearch[®] (upper panel), and the epitope-independent and size exclusion system ParsortixTM (bottom panel)

ses. Unlike previous studies, this work showed that the cell lines within the CTC clusters cooperated on the development of metastases and that tumor cells with lower metastatic potential acquired higher metastatic capability when grouping together [71].

7.4 Origin of CTC Clusters

An important question still under debate is the origin of CTC clusters. Mainly two models are under evaluation; (i) CTC cluster can be directly derived from the primary tumor due to the cohesive unit of tumor cells in an orchestrated phenomenon where tumor cells cooperate and collectively migrate, and (ii) CTC cluster can arise from the aggregation and proliferation of individual CTCs in the bloodstream (Fig. 7.2).

Two previously mentioned studies in breast cancer experimentally addressed this question. By injecting breast cancer color-tagged tumor cells into mice at two different locations (mammary fat pads at opposite flanks), they were able to prove that intravascular aggregation of individual CTCs was not the cause of CTC cluster formation, supportive of the existence of a mechanism of collective cell migration and shedding of CTC clusters into the circulation from the primary tumor [6, 46]. Evidences in support of this have been also shown for pancreatic cancer [45]. The molecular mechanisms linked to the formation of clusters, at least in breast cancer, are connected to two proteins, plakoglobin (JUP) and keratin 14 (KRT14), found to be critical for CTC cluster formation. Both proteins are associated with desmosomes and hemidesmosomes, and involved in cell-cell junctions, necessary for the maintenance of the integrity of CTC clusters. In the same line of thought, experimental evidences gathered on an in vitro platform mimicking the bloodstream have shown that the unfavorable conditions present in the bloodstream will not support the intravascular aggregation and proliferation of individual CTCs [72].

However, a recent report developed with PDXs mouse models bearing metastatic breast cancer showed evidences for the presence of clustered tumor cells both in migration and circulation as the result of aggregation of individual tumor cells rather than collective migration and cohesive shedding to the bloodstream [63]. Using intravital multiphoton microscopic imaging, it was shown that cells expressing the stem cell marker CD44 are capable of aggregating into clusters in the circulation or lung vasculature, and that this marker is required for the formation of metastases. This evidence goes in agreement with an earlier study showing the formation of multicellular aggregates at the sites of their primary attachment to the endothelia previous to metastases formation [73], although intravascular cell proliferation of individual CTCs attached to the endothelium has also been reported as an initial step for lung metastasis formation, without need of extravasation and tissue parenchyma invasion [74]. All evidences point towards a possible combined action of both mechanisms in the formation of CTC clusters, and it allows to speculate about the existence of an interplay or even synergy between both mechanisms [63].

A third model for the origin of CTC clusters has also been recently proposed, called "cell jamming". According to this model, the increasing confinement from the growing mass of tumor or higher density of extracellular matrix (ECM) may promote grouping of the cells, and therefore facilitate CTC cluster formation [75]. This hypothesis or model is supported by in vitro evidences showing that ECM density affects how tumor cells invade. Thus, when ECM density is high, mesenchymal tumor cells show a preference for collective invasion, and single cell invasion is observed under low ECM density conditions [76].

7.5 CTC Clusters Isolation Technologies

Technologies developed for the capture of CTCs could be in principle also applicable for the capture of CTC clusters. However, in almost all cases they have not been designed with this specific purpose in mind, which translates into a low efficiency of recovery, inability to separate CTC



Fig. 7.2 Models of CTC cluster formation. CTC cluster can either derive from groups of tumor cells detaching from the primary tumor and collectively migrating and

intravasating, or can arise from the aggregation and proliferation of individual CTCs in the bloodstream

clusters from single CTCs, and often causing cluster damage and break up during separation. Therefore the main challenge it is not to separate CTC clusters from blood cells but to separate them from individual CTCs without affecting their integrity. From the research point of view, a platform for the isolation of CTC clusters should be able to isolate clusters of different sizes in an epitope independent manner, with short processing times, and able to preserve the integrity of the clusters as well as the recovery of viable cells; but from the clinical standpoint, such a platform should demonstrate reproducibility and clinical validity. A summary of the technologies used for CTC clusters isolation and detection is shown in Table 7.2. CTC clusters are usually small groups of cells, from 2 to 19 cells [44], although CTC clusters bigger than 100 cells have been reported. In this sense, size exclusion methods are the best approach to isolate CTC clusters, yielding a good recovery. In particular, filtration technologies are popular given their easy use and high throughput. However, given the physical properties of CTC clusters, those strategies that exclusively rely on size-based separation might loss a significant fraction of CTC clusters [24]. It is because of this that researchers have devoted efforts to improve and develop technologies for the detection of CTC clusters, mainly combining microfluidics with size exclusion approaches. An example, it is the development of the Cluster-Chip, a microfluidic device designed specifically to capture CTC clusters from whole blood [44]. The Cluster-Chip uses triangular micropillars arrays forming bifurcating traps for the capture of clusters, without compromising their integrity. This chip detected CTC clusters in 30-40% of patients with metastatic breast or prostate cancer, or melanoma; however, it showed some limitations regarding the recovery of the clusters immobilized on micropillar arrays. In response to this problem, the inventors have developed a new microfluidic device relying on a two-stage deterministic lateral displacement (DLD) approach [77]. This system sorts cell clusters based on size and asymmetry, and allows for a high recovery efficiency of viable cells with minimal cluster dissociation. This system remains to be tested in the clinical

setting with cancer patient blood samples. An in detail discussion and revision of methodologies used form CTC clusters isolation can be found in the following reference [24].

7.6 Metastatic Features of CTC Clusters

In addition to the preclinical evidences published in the 1970s [10, 15–17], more recent studies mainly developed in breast cancer have demonstrated the high predisposition of CTC clusters to generate distant metastases than single CTCs. There are strong evidences in support of the high metastatic potential of CTC clusters as compared to individual CTCs. Despite of the reported low frequency of CTC clusters both in the blood of breast cancer patients and in the blood of breast tumor mouse models, it has been shown that CTC clusters are responsible for seeding between 50 and 97% of metastatic tumors in mouse models [6, 46]. Aceto et al. using a mouse xenograft model of MDA-MB-231 LM2 cell line, have reported that CTC clusters have an estimated metastatic potential 23-50 times higher than single CTCs [6]. Interestingly, this work has shown the self-seeding potential of CTC clusters within the primary tumor, as well as their oligoclonal origin. In the same way, and making use of the Confetti and Rainbow mice MMTV-PyMT model, Cheung et al. have estimated the metastatic potential of clusters to be >100 times increased relative to single cells [46]. These two studies support the formation of clusters of tumor cells at the primary tumor and their shedding into the bloodstream as a group. Similarly, a study by Liu et al. using triple negative patient-derived breast cancer models (PDXs) shows that CTC clusters have a higher efficiency in mediating metastasis formation than single CTCs [63]. Interestingly, evidences for a higher efficiency of CTC clusters than single CTCs in forming metastases have also been found in pancreatic cancer and colon cancer models [45, 89].

Despite of the demonstration of the increased metastatic potential of CTC clusters compared to individual CTCs, and the frequent polyclonal

		Markers used for		
Technology	Method	identification	Sample tested	References
High throughput	Enrichment free method;	CKs	Patients' blood	[4, 78]
microscopy for	RBCs lysis followed by		samples	
immunofluorescence	fixation and slide staining			
Carcinoma Cell	Magnetic enrichment with	Pan-CKs	Patients' blood	[28]
Enrichment Kit and MS columns	CK7/8 beads		samples	
ISET®	Filtration based method	α-fetoprotoprotein; EpCAM, CKs, EGFR; TGF-βRI, MMP-2; HER2, plakoglobin	Patients' blood samples	[30, 32, 35, 36, 47, 54, 55, 57, 65]
HBCTC-Chip	Microfluidics	EpCAM, HER2, EGFR	Patients' blood samples	[6, 33]
ScreenCell®	Size based filtration	CKs	Patients' blood samples	[79]
CellSearch®	EpCAM-based immunomagnetic detection	СК 8/18/19	Patients' blood samples	[5, 30, 35, 42, 43, 50, 51, 63, 80]
^{neg} CTC-iChip	Size based microfluidics separation	CKs	Patients' blood samples	[81, 82]
Microcavity array (MCA) system	Microfluidics chip with size and geometry control microcavities for size based separation	Pan-CKs	Tumor cells spiked in blood patients' blood samples	[39]
Spiral microfluidic device	Size based microfluidics separation	CKs	Tumor cells spiked in blood and patients' blood samples	[40, 49]
Vitatex cell-adhesion matrix (CAM) platform	Ficoll density gradient followed by adhesion to CAM coated chamber slides	EpCAM and PSMA	Patients' blood samples	[83]
CMx [®] platform	EpCAM- based affinity	СК20	Tumor cells	[48, 53]
	capture microfluidic		spiked in blood	
	platform		and patients' blood samples	
Deterministic lateral	Size and asymmetry based	None (fluorescently	Tumor cells	[77]
displacement (DLD)	filtration	labelled tumor cells)	spiked in blood	
based two-stage				
continuous flow device				
Flexible micro spring array, FMSA	Size based filtration	CK 8/18/19, Vimentin	Tumor cells spiked in blood and patients' blood samples	[41]
Cluster-Chip	Size based microfluidics separation	Wide-spectrum CKs; NG2, CD146, TYRP-1, αSMA; wide-spectrum CKs, PSA	Tumor cells spiked in blood and patients' blood samples	[44]
Parsortix TM	Size and deformability based microfluidics separation	EpCAM, HER2, EGFR	Patients' blood samples	[61, 62, 84]

Table 7.2	Technologies for CTC clusters isolation and detection
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(continued)

Tachnology	Mathod	Markers used for	Sampla tastad	Pafaranaas
Flow cytometry-based platform	Acoustic cell washing and focusing prior to sorting	None (fluorescently labelled tumor cells)	Tumor cells spiked in mouse blood and blood from a pancreatic cancer mouse model	[85]
Biotin-doped Ppy- deposited microfluidic chip	Conducting polymer- deposited microfluidic platform combined with affinity capture	EpCAM, TROP-2, EGFR, vimentin, and N-cadherin	Patients' blood samples	[56]
3D scaffold chip	Size based and EpCAM based separation	CKs	Tumor cells spiked in blood and patients' blood samples	[86, 87]
CellSieve TM	Size based filtration	Vimentin	Blood from a Ewing sarcoma mouse model and patients' blood samples	[58]
OncoBean Chip	Cell capture by affinity at high flow rates	EpCAM, CK 7/8	Patients' blood samples	[59]
NE-iFISH	Negative enrichment immunofluorescence combined with an in situ hybridization system	CK18	Patients' blood samples	[60]
Multi-flow microfluidic (MFM) system	Based on inertial migration of cells flowing in microchannels	CK 8/18/19	Tumor cells spiked in blood and patients' blood samples	[49, 88]
Microfluidic chips	EpCAM-based immunocapture	CKs	Patients' blood samples	[52]

Table 7.2 (continued)

 $\alpha SMA \alpha$ -smooth muscle actin, *CD146* cluster of differentiation 146, *CK* cytokeratin, *CMx* cells captured in maximum, *EB1* Microtubule-associated protein RP/EB family member 1, *EGFR* Epidermal growth factor receptor, *EMT* Epithelial to mesenchymal transition, *EpCAM* Epithelial cell adhesion molecule, *HER2* Human Epidermal Growth Factor Receptor 2, *ISET* Isolation by SizE of Tumor cells, *MMP-2* matrix metalloproteinase-2, *NE-iFISH* Negative Enrichment Immunofluorescence and an In Situ Hybridization System, *NG2* Neuron-glial antigen 2, *PSA* Prostate-specific antigen, *PSMA* prostate-specific membrane antigen, *TGF-βRI* Transforming Growth Factor-β Receptor Type 1, *TROP-2* trophoblastic cell-surface antigen 2, *TYRP-1* Tyrosinase-related protein 1

seeding occurring from the primary tumor to secondary sites suggesting that different clonal combinations in the cluster could have different properties with respect to growth, it still remains under debate whether the tumor cells within a CTC cluster harbor different metastatic potentials. In support of this, a study in melanoma showed that tumor cells with lower metastatic potential can acquire a higher metastatic capability when grouping together with cells with a higher metastatic potential [71]. On the other hand, it was previously reported, that when injecting melanoma cells with different metastatic properties as cellular aggregates, the presence of metastatic cells did not change the inability of non-metastatic cells to proliferate in a distant organ [90]. This last piece of evidence suggests that the metastatic potential of a CTC cluster may depend on the most malignant tumor cells. Further experimental evidences are needed in order to determine whether cooperation between heterogeneous clones making up tumor cell clusters is really happening, and also what is the significance for the metastatic potential of CTC clusters. At this point, it is worth reminding that it is now well accepted and demonstrated the existence of heterogeneous populations of CTCs, with a differential contribution to the metastatic process in prostate, lung and breast cancer [91–93]. In this regard, CTC cluster show both epithelial and mesenchymal traits at the same time, in breast cancer and other tumor types [38, 94]. CTC clusters isolated form breast cancer patients have been found to be positive for mesenchymal markers such as fibronectin, N-cadherin or PAI-1 and weakly positive for endothelial markers (EpCAM or cytokeratins). These findings could indicate a possible cooperative behavior between mesenchymal CTCs and epithelial CTCs within the same cluster, although it has not been formally probed. In order to address this question, cells from an individual CTC clusters should be individualized and analyzed at single cell level, proving the existence of a heterogeneous population of CTCs expressing either epithelial markers or mesenchymal markers.

7.7 Survival and Proliferative Advantage of CTC Clusters

Metastasis is regarded as a highly inefficient process. The vast majority of tumor cells shed into the bloodstream do not survive. It is only a small fraction of CTCs that are viable and capable of surviving, seeding distant organs, and eventually giving rise to overt metastatic disease. This argues that only those CTCs able to survive the transit in the bloodstream will stand a chance in order to contribute to the development of metastases.

CTC clusters have a survival advantage over single CTCs, and we nowadays partially understand some of the underlying reasons. An important feature of the CTCs forming the clusters is that they have strong cell–cell contacts linking them together [95]. It is well established that loss of adhesion-dependent survival signals by epithelial cells when transitioning in the bloodstream leads to anoikis, being therefore causative of CTCs death [96]. This goes in support of the idea that strong cell–cell interactions in the clusters can provide survival stimuli favoring their metastatic spread [75, 97]. Indeed, the interaction between the proteins circulating galectin-3 and cancer-associated mucin1 (MUC1), as well as CD44-mediated signaling pathways, promote homotypic tumor cell aggregation and cluster formation, and prevents CTCs in circulation from anoikis in breast and colon cancer [63, 98], enhancing metastases formation potential.

CTC clusters seem to have a shorter half-life in circulation than single CTCs (6–10 min and 25–30 min, respectively) [6], what may help them to survive favoring the outgrowth into micrometastases [99]. Mouse studies in breast cancer showed that CTCs clusters are more resistant to apoptosis at distal metastatic sites than individual CTCs, allowing them to expand more rapidly. Thus, disseminated tumor cells in the lungs of mice injected with CTC cluster did not undergo apoptosis, opposite to disseminated cells from mice injected with single cells which underwent massive apoptosis [6].

The protection of CTC clusters against apoptosis was also shown in patients with small-cell lung cancer; while a 57% of patients showed apoptotic single CTCs (from 0.2 to 20% of CTCs), none of the patients presenting CTC clusters have apoptotic cells within the clusters [5]. Likewise, a study of triple negative breast cancer patients found that only a 0.4% of the cells in the clusters (4 cells out 943 in a total of 194 clusters) were apoptotic, as opposed to a 20% of apoptotic single CTCs (1674 cells out of 8393 single CTC) [43]. These clinical evidences clearly support the protection of CTCs forming the clusters to apoptosis.

Other factors possibly modulating the survival of CTC clusters while transitioning in the bloodstream have been proposed. In patients with MBC the hybrid epithelial-mesenchymal phenotype observed in CTC clusters, which confers a substantial plasticity to these aggregates, has been put forward as a feature for survival advantage [38]. Mesenchymal traits favoring a migratory phenotype together with the preservation of cell–cell junctions of epithelial cells, seem to be the underlying mechanism [100]. Furthermore, methylation and gene expression analyses in CTC clusters from both breast cancer patients and breast cancer xenograft models revealed an enrichment on genes related to cell-cell junction, proliferation and DNA replication [61]. Indeed, CTC clusters show an increase in the percentage of CTCs expressing the marker Ki67 compared to single CTCs, indicative of a higher proliferation rate. Also, CTC clusters seem to share several properties that commonly feature stem cell biology [61, 63]. These features may play a relevant role in the intravasation, enhanced adaptation to new microenvironments and facilitate metastasis initiation by CTC clusters. Interestingly, the epigenetic signature found in CTC clusters, hypomethylated regions enriched with embryonic stem cell transcription factor binding sites, correlates with an enhanced metastatic phenotype and with poor prognosis in patients with breast cancer [61].

7.8 CTC Clusters, a Small Portion of Tumor Microenvironment

It has been suggested that the presence within the CTC clusters of immune cells, platelets and stroma-derived cells and factors, known as heterotypic clusters, may be of benefit for the survival and metastatic outgrowth of CTC clusters [100] (Fig. 7.3). Although the role of tumor microenvironment components within CTC cluster remains largely uncharacterized, some evidences are starting to emerge.

Platelets coating CTCs and CTC clusters in the bloodstream act as a physical shield protecting them from the shear forces [101] and immune attacks [102], but also protecting them through the paracrine secretion of factors such as transforming growth factor β (TGF- β), a known inducer of EMT [38]. Staining of CTC clusters isolated from the blood of MBC patients showed an abundance of attached platelets, what goes in support of the strong TGF- β signatures found in mesenchymal CTC clusters [38].

Cancer associated fibroblasts (CAFs) similarly to cancer cells, can disseminate through the circulation to secondary sites, suggesting a role for these cells in the metastatic process [103]. Indeed, the presence of CAFs in heterotypic CTC clusters enables an enhanced survival of tumor cells and also provides growth advantage to them after seeding at distant sites. This has been proved in an experimental setup in mice in which depletion of fibroblasts in the clusters reduced their capacity to form lung metastases [104]. However, even though it has been suggested that CAFs promote tumor growth and metastasis, new evidences also support antitumor actions; meaning, at least, that the role of CAFs within heterotypic CTC clusters need to be further investigated.

Among the white blood cells (WBC) found forming clusters with CTCs, neutrophils seem to play an important role on CTC clusters mediatedmetastasis [84]. The direct interaction of neutrophils with breast cancer CTCs shapes the transcriptional profile of tumor cells supporting cell cycle progression in circulation and accelerating metastasis seeding. Moreover, neutrophils are actively involved in the genesis of CTC clusters, as their depletion in BC animal models reveled a delayed shedding of CTCs and CTC-neutrophil clusters from the primary tumor, a delayed metastasis development, and a shorter overall survival of the mice. Of note, those BC patients in whom at least a single neutrophil-containing CTC cluster was found had a worse progression-free survival than patients with ≥ 5 CTCs in 7.5 ml of peripheral blood [84].

7.9 Prognostic Value of CTC Clusters in Metastatic Breast Cancer

Enumeration of CTCs by CellSearch[®] platform has been extensively proved to be an independent predictor of survival in patients with MBC [105, 106]. Importantly, the prognostic value of CTCs has also been proved in patients with early breast cancer [107]. Despite the demonstration of the prognostic value of CTC enumeration in breast cancer, it took a decade to demonstrate the prognostic value of CTC clusters (A summary of studies in breast cancer in which the prognostic value of CTC clusters has been investigated is



Fig. 7.3 CTC clusters can exist as homotypic or heterotypic entities. Heterotypic CTC clusters seem to have an enhanced metastatic potential compared to homotypic CTC clusters. The presence of stroma and immune cells within the clusters provides survival and growth advantages to CTCs

Breast cancer stage and subtypes	Findings	Enrichment method	References
Stage IV, all subtypes (n = 79)	Patients with CTC clusters across more than three time points had a shorter mean PFS.	^{HB} CTC-Chip	[6]
Stage IV, triple-negative breast cancer (n = 60)	No difference in PFS at baseline but presence of CTC cluster at day 15 and day 29 was associated with shorter PFS.	CellSearch®	[43]
Stage III–IV, all subtypes (n = 115)	Patients with CTC clusters at baseline before first-line therapy had a shorter PFS. CTC clusters might provide additional prognostic value compared with CTC enumeration alone.	CellSearch®	[42]
Stage IV, all subtypes (n = 52)	No difference in PFS at baseline. Shorter PFS and OS for patients with CTC clusters during treatment. CTC clusters may offer additional prognostic information to enumeration.	CellSearch®	[51]
Stage III–IV, all subtypes (n = 128)	CTC cluster enumerations at baseline and during follow-up independently predicts disease progression and overall survival. CTC clusters add additional prognostic value compared with CTC enumeration alone.	CellSearch®	[50]
Stage IV, all subtypes (n = 156)	Shorter PFS and OS at baseline and after treatment. Longitudinal evaluation of CTC clusters improves prognostication and monitoring in patients starting first-line systemic therapy. The presence of CTC clusters adds significant prognostic value to CTC enumeration alone.	CellSearch®	[80]
Stage IV, all subtypes (n = 118)	Worse OS of patients with detectable CTC clusters versus patients with single CTCs only	CellSearch®	[63]

Table 7.3 Clinical studies evaluating the prognostic value of CTC clusters in breast cancer

PFS progression-free survival, OS overall survival

shown in Table 7.3); although initial evidences for this were previously shown in liver cancer and small-cell lung cancer [5, 32].

A prospective randomized phase II trial determined the number of CTC clusters and evaluated its predictive value in a cohort of 32 metastatic Triple Negative Breast Cancer patients (TNBC), on samples collected at baseline, and follow-up after initiation of therapy. This work demonstrated that the persistent presence of CTC clusters detected by CellSearch® at follow-up, but not baseline, was associated with shorter patient survival [43]. This goes in agreement with a previous study in patients with small-cell lung cancer (SCLC) showing that the presence of CTC clusters was significantly associated with worse prognosis [5]. In addition, it was previously shown that the presence of CTC clusters in patients with progressing metastatic breast cancer (79 patients) also correlates with poor prognosis, although in this occasion the technology used for CTC cluster identification was the

^{HB}CTC-Chip [6]. This chip has a high efficiency capturing both small and large clusters [33], it isolates CTC clusters based on the expression of EpCAM, HER2, and the mesenchymal marker CDH11. Interestingly the study included patients with different breast cancer subtypes, and showed that the persistent presence of CTC clusters in the blood of these patients was associated with an adverse clinical outcome [6]. Likewise, the authors reproduced these data on a cohort of prostate cancer patients. Taken together, these studies demonstrate the prognostic value of CTC cluster in advanced breast cancer regardless the technology used for their identification.

Furthermore, in recent years a few other studies have evaluated and corroborated the prognostic value of CTC clusters in breast cancer. It is important to notice that in all following studies the presence of CTC clusters was evaluated using the CellSearch[®] platform. Indeed, these studies have shown that CTC cluster evaluation added additional prognostic value to CTC enumeration alone [42, 50, 51, 80]. Thus, a prospective study involving 115 advance breast cancer patients (stage III and IV), from all subtypes, has shown that CTC cluster evaluation allows for the stratification of patients with elevated baseline CTCs into different survival groups [42]. It also reported that the prognostic value of CTC-clusters appeared to be more pronounced in patients with inflammatory breast cancer, and showed evidences for a yet unreported worse prognosis for patients with CTC clusters present at baseline. A latter work in a cohort of 52 MBC patients from all subtypes undergoing first-line systemic therapy, also showed a poorer prognosis in terms of progression-free survival and overall survival for those patients in which CTC clusters were present in peripheral blood during treatment [51]. This effect was independent of other prognostic factors such as CTC numbers and breast cancer subtype. Similarly, a study in a cohort of 156 MBC patients starting first-line systemic therapy, including all subtypes, showed that longitudinal evaluation of CTC clusters improves prognostication and monitoring. Again this work indicates the added prognostic value of CTC clusters to CTC enumeration alone, and showed no association between breast cancer subtype and presence of CTC clusters [80]. On the other hand, the prognostic value of CTC clusters at baseline is still under debate with evidences building up in both senses [42, 43, 50, 51, 80].

In addition, these clinical studies are also shedding light on the biology of CTC clusters. Thus, a link between CTC cluster size and patient prognosis has been established [50]. Longitudinal data collected from 128 MBC patients at baseline and before starting a new therapy revealed that patients with CTC clusters composed of 3 cells have a pronounce decrease in OS compared to patients with 2-cell CTC clusters. These findings are in line with preclinical evidences previously reported [10, 15]. Moreover, evaluation of the expression of the stem cell marker CD44 in CTC clusters showed that patients with CD44⁺ CTC clusters had a lower OS than patients with CD44-CTC clusters [63]. Finally, these studies indicate that CTC cluster are more often found in TNBC and HER-2 positive patients than in hormone receptor-positive patients [43, 51].

In summary, these studies clearly demonstrate that CTC cluster counts it is an independent prognostic factor, as the presence of CTC clusters adds significant prognostic value to CTC enumeration alone in patients with high CTC counts.

7.10 Therapeutic Implications: Targeting CTC Clusters

Given the importance of CTC cluster to the development of metastasis, research efforts are being directed to identify possible vulnerabilities of clusters in order to target them. In this sense, the advancement on the knowledge of the biology of these cells through their molecular phenotyping is crucial to find or design specific treatments. In this regard a few *proof of concept* studies have been published.

The identification of plakoglobin as a gene highly overexpressed in CTCs from clusters relative to single CTCs as well as its expression in primary breast tumors associated with a significantly reduced distant metastasis-free survival led to investigate its potential as a therapeutic target. Knockdown of this gene in breast cancer cell lines injected into mice led to a diminished presence of CTC clusters in the blood as well as a decreased metastasis formation, suggesting that is a key mediator in tumor cell clustering, without altering primary tumor growth [6]. Similarly, keratin 14 has also been identified to be highly enriched in some CTCs of the clusters as well as in micrometastases, relative to primary tumors or macrometastases [46]. In this case, the knockdown of keratin 14 in the primary tumor led to a decrease in metastasis formation. As both proteins, plakoglobin and keratin 14, are involved in cell-cell junctions necessary for the maintenance of the integrity of CTC clusters, a link can be established between cluster integrity and metastasis seeding, suggesting that the disruption or disaggregation of CTC clusters could be a valid therapeutic strategy. This idea is further supported by recent data showing that the knockdown of CD44 or the use of an anti-CD44 neutralizing antibody disrupted tumor cell aggregation and diminished metastasis formation by CTC clusters [63].

In this sense, the treatment of breast tumor bearing mice with the thrombolytic agent urokinase, exerted and antimetastatic effect by dissociating CTC Clusters [108]. Importantly, these mice showed a 20% increase in survival upon urokinase treatment relative to control animals. More recently, a screening for compounds able to dissociate CTC clusters found that Na+/K+ ATPase inhibitors can efficiently reduce cluster size [61]. Further analysis of the Na+/K+ ATPase inhibitor ouabain in a breast cancer model, showed that the administration of this compound to mice is able to in vivo suppresses the ability of tumors to shed CTC clusters (while increasing the frequency of single CTCs), leading to a remarkable reduction on overall metastasis formation.

As previously mentioned, heterotypic CTC clusters may have enhanced metastatic potential as to that of homotypic clusters, suggesting that the targeting of stromal components within the clusters might be a successful strategy to limit the metastasis seeding capacity. An initial indirect indication for this showed that the depletion of CAFs, which spontaneously metastasize along with cancer cells, in a metastasis mouse model of lung cancer, reduced the number of lung metastases [104]. More recently, it has been shown that the molecule VCAM1 has an important role in mediating the interaction between CTCs and neutrophils, and that the targeting of this molecule prevents the formation of CTC-neutrophil clusters which have an enhanced metastasis seeding capacity [84].

Although so far limited in number, these evidences support a model by which targeting CTC clusters could be a valuable therapeutic approach. Indeed, they support two possible different therapeutic strategies that could be of benefit for cancer patients (at least in breast cancer), i) Preventing CTC cluster formation at early stage for the treatment of cancer while a localized disease and before it disseminates (neoadjuvant and adjuvant treatment), and ii) Disassembling of CTC clusters while in circulation for the treatment of late disease stages cancer to prevent metastasis from seeding other metastases.

7.11 Remaining Questions and Opportunities

Based on current evidences, CTC clusters seem to be responsible for the formation of tumor metastasis. Despite of their origin, whether they are formed by collective shedding to the blood stream or by intravascular aggregation, these tumor cell aggregates have and enhanced survival capacity and improved secondary tumor growth. Interesting features are now known about the biology of CTC clusters; i.e. a hybrid epithelialmesenchymal profile, a stemnes phenotype, and heteroptypic composition. Most importantly, these features are being correlated to a worse prognosis in breast cancer patients, suggestive of the many clinical implication of CTC clusters. But this knowledge raises important questions that needed to be answered. It remains to be determined whether the oligoclonal/polyclonal nature of CTC clusters is the result of an oncogenic cooperative behavior between tumor subclones. Whether CTC clusters hold tumor cells with diverse molecular phenotypes conferring a differential metastatic capacity. It is yet elusive whether the hybrid epithelial-mesenchymal phenotype observed in CTC clusters is due to the combination of cells with a heterogeneous EMT phenotype or rather a mixture of cells bearing either epithelial or mesenchymal features. If the later, evidences are needed of a cooperative behavior between mesenchymal CTCs and epithelial CTCs within the cluster. Moreover, finding out the specific influence of other cell types, such as tumor-associated macrophages, fibroblasts, or leukocytes, on the CTCs within a heterotypic cluster, grants further mechanistic investigation. In this sense, technological aid is paramount. The advancement on the development of more efficient CTC clusters isolation technologies and their combination with under development single cell genomic, transcriptomic, and proteomic analyses is key to address these important questions.

The increased knowledge on the biology of CTC clusters brings about new therapeutic opportunities to interfere with the process of metastasis. Current experimental evidences indicate that CTC cluster disaggregation, as a therapeutic approach, seems quite plausible. However, this strategy may entail some risks (at least for urokinase treatment), since it may increase the invasiveness of tumor cells and therefore metastatic spreading, resulting in the opposite effect [109]. Alternatively, interfering with non-tumor cells associated to CTCs in the clusters may provide a new therapeutic approach, as recently showed [84]. But in this case, more mechanistic insights on how these cells affect tumor cells during different steps of metastasis are needed. Lastly, further knowledge about what are the therapeutic implications of tumor cell clusters, remains to be acquired. CTC clusters represent a challenge because they could contain tumor cells with different drug uptake and resistance properties [110, 111], and even it is now suggested that cluster "compactness" may predict early treatment response in different cancer types including breast cancer [112]. Emerging methods for the ex*vivo* culture of CTCs are very valuable tools for the assessment of drug response and resistance, but they will also help to address some of the question mentioned above.

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Epigenetics of Circulating Tumor Cells in Breast Cancer

Aida Bao-Caamano, Aitor Rodriguez-Casanova, and Angel Diaz-Lagares

Abstract

Liquid biopsy based on the analysis of circulating tumor cells (CTCs) has emerged as an important field of research. Molecular characterization of CTCs can provide insights into cancer biology and biomarkers for the clinic, representing a non-invasive powerful tool for monitoring breast cancer metastasis and predict the therapeutic response. Epigenetic mechanisms play a key role in the control of gene expression and their alteration contributes to cancer development and progression. These epigenetic modifications in CTCs have been described mainly related to modifications of the DNA methylation pattern and changes in the expression profile of noncoding

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A. Rodriguez-Casanova Cancer Epigenomics, Translational Medical Oncology (Oncomet), Health Research Institute of Santiago (IDIS), University Clinical Hospital of Santiago (CHUS/SERGAS), Santiago de Compostela, Spain RNAs. Here we summarize the recent findings on the epigenetic characterization of CTCs in breast cancer and their clinical value as tumor biomarkers, and discuss challenges and opportunities in this field.

Keywords

Epigenetics · DNA methylation · Noncoding RNAs · Circulating tumor cells · CTC · Liquid biopsy · Breast cancer

8.1 Introduction

Breast cancer is the most common tumor diagnosed in women, with 2.1 million newly diagnosed cases in 2018, and it is the main cause of cancer death in females worldwide [1]. Although

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deaths related to this type of tumor have decreased in last years, in part due to the early diagnosis, unfortunately some patients present distant metastasis at the time of diagnosis reducing the possibilities of effective therapy [2]. Breast cancer is considered a multifactorial disease where there is an association with several factors including environmental, hormonal, genetic and epigenetic, diet and lifestyle [3, 4]. According to the gene expression profile, it can be classified into different subtypes and it has been described as a complex and heterogeneous disease with distinct clinical behavior and histopathological features [5, 6].

Although there are some circulating biomarkers (e.g. CA15.3 or BR27.29) to evaluate breast cancer, due to their low sensitivity [7] it is necessary to find new non-invasive biomarkers and mechanisms for the evaluation and characterization of breast cancer. In this sense, in recent years liquid biopsy has emerged as a very important non-invasive tool useful for the clinic and the characterization of tumors [8]. Liquid biopsy refers to the analysis of circulating material in biological fluids that comes from tumors. This methodology incorporates great advantages to the clinical practice, since it allows with high sensitivity and specificity a non-invasive detection of the tumors, the monitoring of therapy response, quantification of minimal residual disease and evaluation of the development of resistances to therapy [9, 10]. Among the tumor material that can reach bloodstream containing tumor-derived information we can find circulating tumor cells (CTCs), circulating DNA (ctDNA), circulating noncoding RNAs (ncRNAs) and microvesicles like exosomes [11–14].

In recent years CTCs have emerged as an important field of cancer research with great implications in cancer progression and metastasis of different tumors, including breast cancer [15, 16]. CTCs are rare cells shed from a primary tumor or metastatic site that circulate through blood to establish in a new tissue to form a metastatic lesion. These cells have variable morphology depending on the cancer type and stage and in blood appear in frequency of 1 or less CTC per 10^{6} – 10^{7} leukocytes depending on the disease stage and aggressiveness of the tumor [17, 18]. In addition, CTCs can appear in circulation as single

cells or clusters of cells (CTC-clusters), which are associated with higher metastatic potential [19]. Nowadays there are different systems to isolate CTCs mainly based on (i) EpCAM based enrichment, (ii) leukocyte depletion and (iii) size-based enrichment [20–22]. Once isolated, CTCs can be enumerated or characterized at molecular level to provide insights into cancer biology and biomarkers for the clinic [15, 23]. One of the molecular mechanisms that can be disrupted in CTCs is the epigenetic machinery, such as DNA methylation and ncRNAs [24, 25]. Epigenetic mechanisms regulate gene expression in different types of cells and conditions [26], showing in cancer an aberrant epigenetic pattern associated with cancer progression and metastasis [27, 28].

In the field of breast cancer, CTCs have shown a key role to evaluate the disease. Thus, the enumeration of CTCs by the CellSearch® system was approved by FDA as a prognostic biomarker for metastatic breast patients [11]. Beyond abundance of cells, different molecular alterations have been evaluated in CTCs as potential biomarkers in breast cancer. These studies have been mainly focused in non-epigenetic molecular mechanisms, however, recent studies have also evaluated the potential of epigenetic marks in CTCs of breast cancer patients [29, 30], showing to be a hallmark of CTCs. Therefore, in this review we provide an overview of the epigenetic mechanisms in CTCs of breast cancer, mainly DNA methylation and ncRNAs, and their implication in tumor progression and metastasis, as well as their value as clinical biomarkers.

8.2 The Epigenetic Machinery: DNA Methylation and Noncoding RNAs

The term epigenetics was first proposed by Waddington et al. in 1942 [31]. Epigenetics refers to hereditary changes in the activity and expression of genes that occur without altering the DNA sequence [32, 33]. This mechanism plays an important role in regulating the gene expression of many biological processes [26]. Epigenetic mechanisms show several levels of regulation (Fig. 8.1): DNA methylation, histone modifications, posi-



Fig. 8.1 Schematic representation of the epigenetic machinery. Epigenetic mechanisms play a key role in the regulation of gene expression of both coding and noncoding genes. In cancer these epigenetic modifications can

tioning of the nucleosome and non-coding RNAs (ncRNAs) [34]. In particular, DNA methylation and ncRNAs are two of the most widely studied epigenetic players with important implications in cancer development and progression [9].

8.2.1 DNA Methylation

The best-known epigenetic mechanism is DNA methylation, which is a covalent modification of the DNA resulting from the addition of a methyl group (CH₃) to the 5' carbon of cytosines in cytosine-phosphate-guanine (CpG) dinucleotides leading to 5-methylcytosine (5mC) [35]. This process is enzymatically regulated by DNA methyltransferase (DNMT) enzymes (DNMT1, DNMT3A and DNMT3B) that catalyze the transference of methyl groups from the S-adenosil-L-metionine (SAM) to the cytosines. The establishment of the DNA methylation profile

be deregulated inducing development and progression of tumors. These epigenetic players can be used as cancer biomarkers for breast cancer and other types of tumors

needs a *de novo* methylation process that is controlled by the enzymes DNMT3A and 3B. On the other hand, the enzyme DNMT1 is responsible for maintaining the methylation patterns during cell division [36, 37]. DNA methylation generally occurs in certain areas of the genome, such as gene promoters, that present a high concentration of CpG dinucleotides defined as CpG islands. However, DNA methylation also occurs in other different genomic regions to maintain the conformation and integrity of the chromosomes, as well as to avoid the potential damage of the mobile genetic elements [38].

DNA methylation mechanism plays an important role in regulating gene expression, which can undergo alterations inducing the development of several diseases, such as cancer [28]. Thus, there are certain regions of the DNA that can gain methylation (hypermethylation) whereas other sequences can loss this methylation mark (hypomethylation) [35]. In cancer, hypermethylation of promoters in CpG islands is usually linked to the silencing of both coding and noncoding genes [39, 40]. However, genome-wide hypomethylation has been associated with the expression of proto-oncogenes, genomic instability and malignant transformation of tumors [41, 42]. In breast cancer there are some studies that have shown the promoter hypermethylation of certain tumor suppressor genes. Some of these epigenetically regulated genes are Ras-associated domain family member 1A (RASSF1A), cyclin D2 (CCND2), glutathione S-transferase P1 (GSTP1), hypermethylated in cancer 1 (HIC1), retinoic acid receptor beta ($RAR\beta$), and death-associated protein kinase (DAPK) [43–48]. For example, the methylation of RASSF1A has been associated to the progression of breast cancer and metastasis development [49]. On the other hand, the methylation of GSTP1 has shown to be related with differential response to chemotherapy and the survival of the patients with breast cancer [50].

It is also important to note that DNA methylation is a reversible epigenetic mechanism that can be reversed in human cells by ten-eleven translocations (TET) enzymes. TET enzymes play central roles in regulating gene expression catalyzing the conversion of 5mC to 5-hydroxy-methylcytosine (5hmC) in several tissues [51]. The function of these enzymes can be altered in cancer leading to an imbalance in genomic 5mC/5hmC levels that is associated with oncogenic transformation, including in breast cancer [52]. Importantly, there are also epigenetic-based drugs (epidrugs) that are able to reverse the methylation status of genes inducing hypomethylation [53]. One example is the group of DNA methyltransferase inhibitors (DNMTi), such the nucleoside analogues 5-azacytidine as (5-AZA-CR) and decitabine (5-AZA-CdR), which were the first FDA-approved epidrugs for the treatment of patients with myelodysplastic syndromes and certain leukemias [54].

8.2.2 Noncoding RNAs

In addition to DNA methylation, noncoding RNAs also play an important role in the control of gene expression [55, 56]. It has been postu-

lated that almost 98% of the transcriptome correspond with noncoding transcripts [57]. These noncoding RNAs (ncRNAs) are mainly classified according to their length using 200 nucleotides (nt) as a cutoff. Thus, we can find small ncRNAs (sncRNAs) with less than 200 nt, including microRNAs (miRNAs), small interfering RNA (siRNA) and piwi-interacting RNA (piRNA). And there also long ncRNAs (lncRNAs) with more than 200 nt, including long intergenic ncRNAs (lincRNAs), long intronic ncRNA (intronic lncRNAs) and circular RNAs (circRNA) [58–60].

Among the sncRNAs, microRNAs (miRNA) are the most widely studied. miRNAs (18-25 nt) are single-stranded molecules that bind to specific regions of target messenger RNA (mRNA) and mediate posttranscriptional gene silencing by blocking transcription or degrading mRNA [61]. Through these mechanisms, a single miRNA can regulate the expression of hundreds of genes regulating important features for cancer tumorigenesis [62]. Therefore, microRNAs in cancer can show tumor suppressor ("suppressor-miRs") or oncogenic ("onco-miRs") properties, where onco-miRs are usually over-expressed whereas suppressor-miRs are downregulated [63, 64]. In addition, miRNA signatures have shown to be specifically associated with different types of cancers leading to define the molecular characteristics of tumors [65].

The number of ncRNAs identified in recent years is increasing rapidly. In particular, it has been recently described that lncRNAs constitute the vast majority of the non-coding transcriptome [66]. Although lncRNAs lack the potential to encode proteins, they may exhibit some mRNAlike properties, such as multiexonic gene structures, polyadenylation, presence of 5' cap and transcription by RNA polymerase II [67, 68]. LncRNAs have important functions controlling gene expression and are associated with a great variety of regulatory functions, such as splicing control and transcriptional regulation [69, 70]. Although most of the lncRNAs have not yet been studied in detail, some of these molecules have been characterized in cancer, showing that they can act as oncogenes (e.g. HOTAIR and MALAT1) [56, 71] or as tumor suppressor genes (e.g. TP53TG1, LED, LINC-PINT) [40, 70, 72].

Both microRNAs and lncRNAs can be deregulated in breast cancer. In 2005 Iorio et al. identified for the first time the disruption of microRNAs associated to breast cancer. In this work they identified the expression of several microRNAs (e.g. miR-125b, miR145, miR-21, and miR-155), associated with relevant characteristics of breast cancer including estrogen and progesterone receptor expression, stage of the disease, invasion or proliferation [73]. Since this study several microRNAs have been identified in relation to different breast cancer subtypes [74], as well as the regulation of stemness [75]. Similarly, some IncRNAs have shown aberrant expression associated to breast cancer tumorigenesis. For example, the oncogenic lncRNA HOTAIR is highly expressed in breast tumors promoting cancer metastasis [56], invasion [76] and cell proliferation [77]. Some other lncRNAs have shown tumor suppressor functions in breast cancer, such as GAS5, which is downregulated in breast tumors inducing proliferation due to the inhibition of apoptosis [78].

8.3 Methods for the Detection of Epigenetic Mechanisms in CTCs

There are a variety of techniques that can be used to detect epigenetic mechanisms either at genome-wide scale or in a specific locus [79–82]. DNA methylation can be analyzed using different approaches based on methods that use bisulfite conversion, restriction enzymes, specific antibodies or nanopore-based single DNA sequencing [83, 84]. Combined with these approaches DNA methylation can be assessed for genome-wide screening with NGS or microarrays systems [40, 85, 86], or for locus-specific assays using different technologies including pyrosequencing, methylation-sensitive high resolution melting (MS-HRM), MethyLight assay, quantitative methylation-specific PCR (qMSP), methylation-specific PCR (MSP) or Methyl-BEAMing, among others [39, 87–91]. On the other hand, the expression of ncRNAs can be detected at transcriptomic level with NGS (RNAseq) and microarrays or by means of the analysis of specific transcripts with quantitative methods such as qRT-PCR [40, 92–94]. Due to the differences between methodologies, it is important to consider their advantages and limitations for the selection of the appropriate option [95].

Some of these well-known technologies have already been used in CTCs (Table 8.1) for locusspecific DNA methylation analysis such as MSP, qMSP, HRM and pyrosequencing [24, 96, 97]. However, other new methodologies to analyze DNA methylation in CTCs are emerging. This is the case of the development of a single-cell protocol based on agarose embedded bisulfite treatment (scAEBS) that allows the analysis of DNA methylation of multiple loci using multiplex PCR (multiplexed-scAEBS) [98]. This method is an adaptation of the agarose embedded bisulfite treatment (AEBS) protocol previously described [99] and it is based on bisulfite conversion singlecell methylation analysis. Importantly, the multiplexed-scAEBS allows the detection of allele-specific methylation in different genes of single CTCs [98].

In addition to specific locus, DNA methylation of CTCs can be analyzed at genome-wide level both with microarrays systems and NGS. In this sense, DNA methylation microarrays were used for the analysis of invasive CTCs (iCTCs) after the isolation of these cells with the Vitatex cell-adhesion matrix (CAM) platform [100]. In addition, NGS after bisulfite conversion of DNA has recently shown to be useful for CTC analysis, allowing the detection of multiple CpGs differentially methylated between single CTCs and CTCclusters [101].

Regarding the analysis of ncRNAs, mainly miRNAs have been analyzed in CTCs. Some of the studies have detected individual transcripts or a panel of specific transcripts using qRT-PCR after the isolation of CTCs with CellSearch® system or immunomagnetic beads [25, 102]. Interestingly, qRT-PCR can also be used after the extraction of miRNAs from CTCs using a Flinders Technology Associates (FTA) Elute Card [103], which is a cellulose paper able to

[25, 102]

[105, 106]

Epigenetic mechanism	Method	Approach	References
DNA methylation	MSP	Target specific	[24, 108]
	qMSP	Target specific	[96]
	HRM	Target specific	[97]
	Pyrosequencing	Target specific	[97]
	Multiplexed-scAEBs	Multiple targets	[24]
	Methylation arrays	Genome-wide	[100]
	NGS	Genome-wide	[101]

Table 8.1 Methods more frequently used for detecting epigenetic mechanisms in CTCs

qRT-PCR

ISH-LNA

MSP Methylation-specific PCR, *qMSP* Quantitative methylation-specific PCR, *HRM* High resolution melting, *scAEBS* single-cell agarose-embedded bisulfite sequencing, *NGS* Next-generation sequencing, *qRT-PCR* Quantitative reverse transcription PCR, *ISH-LNA* in situ hybridization combined with LNA probes, *LNA* Locked-nucleic-acid

Target specific

Target specific

immobilize cells for the extraction of nucleic acids [104]. Due to its high sensitivity this technique could be useful for the detection of miR-NAs in a low number of CTCs [103]. However, other studies have focused on the analysis of miRNAs in CTCs using in situ hybridization (ISH) methodologies. Thus, Ortega et al. developed the first protocol to detect miRNAs in CTCs using ISH (MishCTC) [105]. This method combines the ISH with the immunomagnetic selection of cytokeratins, immunocytochemistry and locked-nucleic-acid (LNA) probes to detect miR-NAs expression in CTCs. Other group was also able to adapt an in situ hybridization (ISH) protocol using LNA probes in combination with the CellSearch® CTC detection system, which allows the detection of miRNA expression in individual CTCs [106]. One of the advantages of these methods is the use of LNA probes, which increases the efficiency of hybridization improving the ability to detect miRNA expression [107].

8.4 Deregulation of Epigenetic Mechanisms in CTCs of Breast Cancer

Several studies have shown (Table 8.2) that tumor suppressor genes can be epigenetically disrupted in CTCs of breast cancer patients [15, 30], suggesting that epigenetics is a hallmark of CTCs. This epigenetic alterations in CTCs have been mainly described related to modifications of the DNA methylation pattern of genes [24, 96, 109] and changes in the expression profile of noncoding RNAs, especially microRNAs [25, 106] (Fig. 8.2). DNA methylation and ncRNA expression may provide insights into the molecular mechanisms of metastasis and epithelialmesenchymal transition (EMT), with important therapeutic implications [110, 111]. This is a very promising field with many classes of epigenetic modifications little or nothing explored in CTCs that could significantly contribute to decipher the mechanisms underlying cancer progression and metastasis [101].

8.4.1 DNA Methylation in CTCs

Chimonidou et al. provided for the first time that tumor suppressor and metastasis suppressor genes can be methylated in CTCs [24], opening new avenues in the field for the study of DNA methylation in CTCs of cancer patients. After isolating CTCs from peripheral blood of metastatic breast cancer patients using an EpCAM immunomagnetical based assay, this group analyzed the promoter methylation status of a panel of three tumor suppressors by methylationspecific PCR (MSP). One of the genes analyzed was cystatin E/M (CST6), which has been described as a tumor suppressor gene in breast cancer [112] inhibiting proliferation, migration and invasion related to breast cancer bone metastasis [113]. The other genes studied were, SRYbox containing gene 17 (SOX17) and breast cancer metastasis suppressor gene 1 (BRMS1),

miRNAs

		Epigenetic		
Gene	CTC approach	approach	Epigenetic alteration and relevance	References
CST6 BRMS1 SOX17	EpCAM immunomagnetical based assay	MSP	CpG methylation. Association with disease stage	[24]
BRMS1	Peripheral bood cytospins	MSP	CpG methylation. Prognostic biomarker	[108]
CST6 ITIH5 RASSF1	Size-based microfilter	Pyrosequencing	CpG methylation. Prognostic biomarker	[150]
ESR1	EpCAM+ CTCs and CellSearch®	qMSP	CpG methylation. Predictive biomarker of therapy response	[23]
miR-200c/141 miR-200b/a/429 CDH1	CellSearch® and FACS sorting	Multiplexed- scAEBS	CpG methylation. Epigenetic regulation of EMT-associated genes	[98]
Binding sites for: OCT4 NANOG SOX2 SIN3A	Microfluidic-based method	NGS	CpG methylation. Different methylation in single CTCs and CTC-clusters. Potential therapeutic target	[101]
Panel of miRNAs	CellSearch®	qRT-PCR	Overexpression. Potential as epigenetic biomarkers	[25]
miR-21 miR-146a Mir-200c miR-210	EpCAM immunomagnetical based assay	qRT-PCR	Overexpression. Potential as epigenetic biomarkers	[102]
miR-10b	CellSearch®	ISH-LNA	Overexpression. Potential as epigenetic biomarkers	[106]

 Table 8.2
 Epigenetic alterations and biomarkers in CTCs of breast cancer

MSP Methylation-specific PCR, *qMSP* Quantitative methylation-specific PCR, *scAEBS* single-cell agarose-embedded bisulfite sequencing, *NGS* Next-generation sequencing, *qRT-PCR* Quantitative reverse transcription PCR, *ISH-LNA* in situ hybridization combined with LNA probes, *LNA* Locked-nucleic-acid, *LNA* Locked-nucleic-acid, *EpCAM* Epithelial cell adhesion molecule

with important tumor suppressor functions in breast cancer through the regulation of Wnt/betacatenin signaling pathway [114] and chromatin remodeling [115, 116], respectively. Importantly, the methylation analysis of these three tumor suppressor genes revealed that CST6, SOX17 and BRMS1 were hypermethylated in CTCs of breast cancer patients [24], which was later confirmed in another work of the same group [117]. In addition, the methylation status of these genes also showed differences between individual patients, indicating that CTCs are characterized by the presence of a heterogeneous methylation pattern [24].

DNA methylation regulates the expression of genes in normal and tumor cells of different types of tumors [42, 118]. However, at this time this issue is not well characterized in CTCs and there

are few studies that have evaluated this association. In breast cancer one work revealed some correlation between the methylation of BMRS1 promoter analyzed by MSP and the protein expression levels [108]. In other type of tumor other study showed a high correlation between the loss of methylation in c-Met promoter and gene expression in a CTC cell line [97].

The study of single cells provides the opportunity to analyze the complexity and heterogeneity of cells [109]. In this sense, a recent work was able to analyze the promoter methylation status of three EMT-associated genes (miR-200c/141, miR-200b/a/429 and CDH1) in individual CTCs of breast cancer patients [98]. Using multiplexedscAEBS they analyzed the methylation status of 159 single CTCs from 11 patients with metastatic breast cancer, evidencing a heterogeneous level



Fig. 8.2 Epigenetic mechanisms in CTCs of breast cancer patients. The CTCs of breast cancer patients undergo alterations of the epigenetic mechanisms, such as DNA methylation and ncRNA expression. These type of epigenetic players can be characterized in CTCs using epig-

of methylation in CTCs, which is in line with previous studies [24].

In different types of cancers, including breast cancer, CTCs can be present in bloodstream as single cells or aggregates of CTCs (CTC-clusters) [19, 119, 120]. In a very recent study the DNA methylation profile of single CTCs and CTCclusters captured by a microfluidic-based method from breast cancer patients and mouse models was evaluated following a genome-wide DNA methylation approach [101]. The analysis in patient derived-CTCs by NGS revealed a different DNA methylation profile between clusters and single cells, representing a potential therapeutic target. Although the global methylation

enomic approaches (genome-wide) or target-specific assays. The identification of aberrant epigenetic profiles can provide insights into cancer biology and render tumor biomarkers and epigenetic therapeutic targets with an important clinical value for breast cancer patients. *mDNA* methylated DNA, *ncRNAs* noncoding RNAs

pattern was similar, they found specific differentially methylated regions in CTC-clusters, showing a hypomethylation pattern in DNA binding sites for transcription factors related to stemness and proliferation (OCT4, NANOG, SOX2, and SIN3A). Importantly, in vitro CTCcluster dissociation into single cells with the individual treatment of CTC cluster-dissociating compounds (ouabain and digitoxin) induced the DNA methylation reprograming resulting on the hypermethylation of binding sites for OCT4, SOX2, NANOG, and SIN3A, which correlated with a decreased expression of their target genes and metastasis burden. These results also suggested that DNA methylation remodeling was due to the failure in cell-cell junctions after the treatment with CTC cluster-dissociating compounds [19, 121]. Altogether these results linked the epigenetic regulation of CTC-clusters with and increased accessibility for transcription factors relevant for stemness and promoting metastasis, opening a new scenario to reduce cancer metastasis.

8.4.2 Non-coding RNAs in CTCs

In breast cancer, CTCs have shown to have alterations in the microRNA expression profile. In this sense, Sieuwerts et al. analyzed the profile expression of microRNAs by qRT-PCR in CTCs isolated with the CellSearch® system from metastatic breast cancer patients collected before starting first-line systemic therapy in comparison with healthy blood donors [25]. With this approach they identified the overexpression of 10 miRNAs in CTCs, highlighting the relevance of microRNAs molecular characterization. This study was performed in a bulk of CTCs, however, the detection of microRNAs in individual CTCs is also possible. For this purpose Gasch et al. adapted an in situ hybridization (ISH) protocol using LNA probes combined with the CellSearch® CTC detection system [106]. With this methodology they were able to analyze the expression of miR-10b in individual CTCs isolated from the blood of metastatic breast cancer patients and other types of tumors. They demonstrated for the first time a heterogeneous expression of microRNAs in CTCs isolated from the same patient. Importantly, the analysis of miR-10b+ CTCs could be important for breast cancer patients due to miR-10b has shown association with the development of metastasis [122].

MicroRNAs are key regulators of gene expression involved in cancer metastasis by means of different mechanisms [123]. In addition to mir-10b, other microRNAs related to metastasis have been shown to be altered in CTCs of breast cancer patients. This is the case for miR-21, miR-146a, miR-200c, and miR-210 whose expression in CTCs of breast cancer patients is deregulated controlling important functions of the multistep metastatic process related to migration and invasion. In a recent study the expression of these miRNAs was analyzed using qRT-PCR in CTCs isolated from 55 metastatic breast cancer patients by anti-EpCAM-coated immunomagnetic beads [102]. Interestingly, all miRNAs showed significantly overexpression in CTCs of metastatic breast cancer patients compared to healthy controls, which offers the possibility of better understanding the biology of CTCs.

8.5 Connection Between Epigenetic Alterations of CTCs and Circulating Nucleic Acids

The molecular profile of CTCs and circulating DNA can both present alterations related to tumor disease [102, 108, 124]. In breast cancer several studies have shown that there is an association between the molecular pattern of CTCs and circulating DNA or ncRNAs. For example, mutations in circulating DNA are able to reflect the heterogeneity observed in single CTCs, providing a reflection of the molecular profile observed in CTCs [125]. In this sense, breast cancer patients have shown concordance and complementary information between molecular alterations of CTCs and circulating nucleic acids [126], suggesting that CTCs could contribute to the release of epigenetic and other molecular alterations to bloodstream of cancer patients [96, 127, 128].

The methylation status of particular genes in CTCs has shown correlation with the methylation level of the same genes in circulating DNA and tumor tissue. In breast cancer this connection has been confirmed analyzing the methylation status of the gene *SOX17*, which was highly methylated in primary tumors, and in matched CTCs and circulating DNA [96]. In particular, this study showed significant correlation between *SOX17* methylation in circulating DNA and CTCs in patients with operable breast cancer after surgical removal of the primary tumor. Other study evaluated the gene *BRMS1*, which is a candidate metastasis-suppressing gene with an important

function in promoting migration and invasion [129]. The methylation analysis of BRMS1 promoter revealed that this gene is hypermethylated in primary tumors of early stage patients and in their corresponding CTC samples, however not in non-tumoral breast tissues [108]. In addition, the methylation status of the genes APC and GSTP1 in circulating DNA correlated with the presence of CTC in the blood of breast cancer patients. Importantly, both methylated DNA and CTC showed association with a more aggressive tumor biology and advanced disease [130]. In line with this, the methylation of other genes in circulating DNA, including RASSF1A and ESR1, was associated with the detection of CTCs in circulation of breast cancer patients [127].

Similar to DNA methylation, there is a connection between the profile of circulating ncRNAs and CTCs. In this sense, the overexpression of metastasis-related miRNAs, such as miR-21, in CTCs of breast cancer patients was associated with the upregulation of these miR-NAs in the corresponding plasma [102]. In other work, Madhavan et al. evidenced for the first time that circulating miRNAs can predict the CTC status of patients with metastatic breast cancer. They identified a panel of circulating miRNAs able to differentiate between metastatic breast cancer patients with presence or absence of CTC in blood, showing potential to evaluate the progression-free and overall survival of metastatic breast cancer patients [131].

8.6 Epigenetic Biomarkers in CTCs

Epigenetic mechanisms can be measured in body fluids and are useful as tumor biomarkers in clinical practice mainly to assess the risk of cancer development, detect the presence of a type or subtype of tumor (diagnosis biomarker), evaluate the risk of relapse or disease progression (prognostic biomarker), predict the response to certain therapies (predictive biomarkers) and follow the response to the treatment (monitoring biomarker) [132, 133]. This type of epigenetic biomarkers has an important role for the implementation of a more personalized medicine and precision oncology in different types of tumors, including breast cancer [84, 134, 135] (Fig. 8.2).

Epigenetic biomarkers have relevant characteristics to be useful as tumor biomarkers for the clinic due to their reliability, sensitivity, stability, frequency and noninvasive accessibility in biological fluids [132, 136]. Until now several epigenetic biomarker candidates have been proposed in breast cancer. For example, some genes (BRCA1 and RAD51C) have been described in association with risk assessment and early-onset sporadic disease [137]. In addition, epigenetic biomarkers have also shown to be useful in breast cancer for detection (e.g. APC, RASSF1A, DAPK1, miR-21/miR-155/miR-365, HOTAIR) [138–140], prognosis (e.g. CpG island methylator phenotype, RASSF1A, miR-21, MALAT1) [141–144] and for evaluating therapy response (e.g. BRCA1, FERD3L and TRIP10 signature, miR-21, miR-125b, HOTAIR) [145–149].

Epigenetic biomarkers in liquid biopsy are especially important for clinical purposes in cancer in part due to the possibility of analyzing noninvasive samples. Until now most of the epigenetic studies in liquid biopsy have focused in circulating nucleic acids. However, the clinical significance of CTCs has also been studied, suggesting that they are surrogate biomarkers of tumor prognosis and may serve to evaluate the response to chemotherapy [29, 125]. In breast cancer patients the hypermethylation of some genes in CTCs has revealed potential as biomarkers (Table 8.2). This is the case of CST6, SOX17 and BRMS1 whose methylation status has shown a positive association with the stage of the disease [24]. Importantly, the methylation levels of BRMS1 promoter in CTCs was also able to provide prognostic information for disease free survival in early breast cancer [108]. In particular, the hypermethylation of BRMS1 was associated with a lower diseasefree survival and worse prognosis, showing a significantly association with a higher incidence of relapses. Similarly, other group identified the methylation status of several genes in CTCs associated with poor progression-free survival (PFS)

in metastatic breast cancer patients [150]. In this work patients with hypermethylation in CTCs of the genes CST6, ITIH5, or RASSF1 showed poor PFS compared to those ones with unmethylated CTCs, which could be useful to identify patients at high risk for disease progression. DNA methylation marks have also showed connection with the therapy response in breast cancer [147]. Thus, the hypermethylation of the gene ESR1 in CTCs was associated with the lack of response to everolimus/exemestane therapy in patients with ER+/ HER2- advanced breast cancer [23]. This result evidence the great potential of epigenetic marks of CTCs to evaluate therapy response in cancer. Although there are currently few studies evaluating the potential of ncRNAs in CTCs as biomarkers for breast cancer, the deregulation of microRNA expression observed in CTCs (Table 8.2) also suggests great potential as epigenetic biomarkers of the disease [25, 106].

8.7 Conclusions and Future Perspectives

The field of circulating tumor cells has emerged in recent years as an important topic in cancer research, with great implications in cancer progression and metastasis of breast cancer and other tumors [11, 101, 151]. The molecular characterization of CTCs can be useful to provide insights into cancer biology and to identify tumor biomarkers for the clinic. Epigenetic mechanisms, such as DNA methylation and ncRNAs, have shown to play an important role in metastasis and have also an important clinical value as biomarkers for the detection, prognosis and the evaluation of therapy response [9]. In addition, epigenetic mechanisms have the potential to be reversed representing interesting targets for cancer therapy [53, 111].

There are several methods that can be used to detect epigenetic mechanisms, however only a few of them have been used for the epigenetic characterization of CTCs in breast cancer. Some of these approaches are useful for detecting DNA

methylation and miRNA expression in CTCs, based on locus-specific assays or genome-wide analyses. Thus, in the field of breast cancer, DNA methylation and miRNAs have shown to be deregulated in association with cancer progression and metastasis. Interestingly, there is an association between epigenetic alterations of CTCs and the corresponding epigenetic profile detected in bloodstream. This connection suggests that CTCs could contribute to the release of tumoral material with epigenetic alterations to the bloodstream of breast cancer patients [96]. This kind of approach represents an important non-invasive tool for the management and therapy of the breast cancer patients. Although there are relevant advances in the field, studies to evaluate the clinical potential of epigenetic biomarkers in the CTC of patients with breast cancer are still lacking.

The epigenetic characterization of CTCs has been mainly focused in the molecular study of DNA methylation and miRNAs. This type of mechanisms has shown great relevance in breast cancer but there are also other epigenetic players that could bring some light on this tumor, including 5hmC, other types of ncRNAs (e.g. lncRNAs and circular RNAs) and epitranscriptomic modifications (e.g. N6-methyladenosine) [152]. CTCs are rare cells in circulation, therefore the development and improvement of single-cell methods and high sensitive technologies is of great importance to address in depth the complexity of epigenetics in CTCs of breast cancer patients. However, despite the number of existing challenges, the research field on epigenetics of CTCs opens a new scenario to elucidate the mechanisms of metastasis and personalize the management of breast cancer patients.

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Circulating Tumor Cells: Applications for Early Breast Cancer

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Abstract

Breast cancer is the most common malignancy among women. Most of breast cancer patients are diagnosed in early stages and will be treated with curative intent. Despite this, some patients will relapse. The identification of patients at high risk remains an important challenge. CTCs can be useful to identify this patients, to assess tumor dynamics and to monitoring therapy. There is definitive evidence on the prognostic role of CTCs in early breast cancer (eBC) but its clinical utility in daily practice is still lacking. We have to take into consideration that the studies published to date

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mainly evaluated the presence of CTC based on the expression of epithelial surface markers. Future studies need to overcome this limitation and important advances in technical methods can assess CTCs and capture the heterogeneity of the tumor landscape. It is also tempting to speculate that CTCs may also provide complementary information on the interplay of tumor cells with the immune system. The combination of different methods to detect tumoral disease by liquid biopsy may provide new ways to personalize in an unprecedented manner the management of patients with eBC.

Keywords

Early breast cancer · Circulating tumor cells (CTCs) · Prognosis · Clinical trials

9.1 Introduction

Breast cancer is the most common malignancy among women, accounting for 2,088,849 of new cancer diagnoses (11.6% of total cancer burden) worldwide [1]. Thanks to important advances in screening and prevention strategies, most of breast cancer patients are diagnosed early and can thus be offered treatment with curative intent [2]. Despite this, some patients will relapse with metastatic spread of the disease months, years or decades

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after the treatment of the primary tumor [3]. The identification of patients at high risk of relapse remains an important challenge in the field. Here, we review the rationale for studying Circulating Tumor Cells (CTCs) in patients with early breast cancer (eBC) and discuss their potential clinical applications.

Potential clinical applications of CTCs in eBC include: (1) identifying patients at risk of relapse; (2) assessing tumor dynamics to characterize the tumor evolution; (3) monitoring therapy efficacy; (4) identifying potential biomarkers for personalized therapy development [4].

9.2 CTCs Biology and Molecular Characterization in Early Breast Cancer

At the time of initial diagnosis, disseminated tumor cells (DTC) can be detected in the bone marrow in 30% of operable breast cancer patients that lack any clinical or histopathological signs of metastasis. Nevertheless DTCs require invasive methods for their detection and its clinical potential is therefore very limited. According to several studies the concordance between CTC and DTC ranged 66–94% [5].

CTCs are tumor cells that depart, actively or passively, from the primary tumor or from a metastatic site. Even during early stages of cancer, tumor cells can disseminate into the circulation at an estimated rate of 10^6 cells per gram of primary tumor per day [6]. In blood circulation, CTCs can travel as single cells, cell clusters or apoptotic bodies [7], and have the ability to disseminate to distant localizations, where they can adapt and survive [8] (Fig. 9.1).

CTCs are a heterogeneous cell population, constituted mainly by differentiated tumor cells but also harboring sub-populations of cells with resistance, self-renewal and/or tumor-initiating capabilities, otherwise known as cancer stem-like cells (CSCs), which may present important phenotypic differences with regard to the main CTC population [9–11].

Furthermore, the functional capabilities of CTCs may vary depending on the disease context. In eBC, for example, mitotic CTCs are very rare, while most of the CTCs of metastatic breast cancer patients actively divide and can be identified at all stages of mitosis [12–14].



Fig. 9.1 CTCs depart from primary tumor and enter in blood circulation. CTCs may undergo phenotypic changes to acquire a survival advantage. In the bloodstream, CTCs

can travel as single cells or as cell clusters together with platelets, neutrophils, and/or other immune cells increasing their metastatic potential

It is estimated that only 0.1% of single CTCs survive more than 24 h in the bloodstream (their half time ranging from 1 to 3 h), and that less than 0.01% of these cells have the ability to produce metastases [4, 15, 16]. These cells must acquire phenotypic changes (e.g. epithelial mesenchymal transition or EMT) that provide a survival advantage in the bloodstream as well as in foreign tissues [9, 17, 18].

Another important biological aspect to consider with regard to the biological behavior of CTCs is that in the bloodstream, they can be present as single cells or form clusters with other blood cells or endothelial cells, forming aggregates with each other and with blood cells through cytoskeletal protrusions supported by α -tubulin (TUB), vimentin (VIM) and Detyrosinated α -tubulin (GLU) [19]. Recently it has been described the importance of Plakoglobin [20] as adhesion molecule to maintain this aggregation of cells that allow them to be protected from the action of immune system, keeping the aggregation of cells and conferring important advantage to survive in bloodstream and to arrive to the metastatic niche [7]. Clustered cells have 23-50fold increased metastatic potential compared with single cells. The study of the cluster circulating tumor cells, is an important point of research. In this scenario, an elegant study recently published by Gkountela et al. reveal in preclinical models a different pattern of methylation between CTCs and Clusters, identifying specifics hypomethylated binding sites for OCT4, SOX2, NANOG, and SIN3A that promote stemness and metastatic dissemination [21, 22].

EMT allows CTCs to survive in blood circulation inducing the loss of both cell junctions and cell polarity, enabling cell motility and assisting CTCs during intravasation into the bloodstream [23]. This process is extremely complex and involves different molecular pathways, ultimately yielding a survival advantage.

CTCs in blood circulation, can interact with all the elements of immune system and platelets. Interestingly, platelets had a main role in metastatic spread in breast cancer [24]. In preclinical models with cell lines, researchers have shown the adhesion of platelets to CTCs surface. This interaction prevents the recognition of CTCs by the immune system. Other important interaction between CTCs and platelets is related with transforming growth factor beta (TGF β) pathway. This signaling pathway, promoted by platelets, assists a process of epithelial to mesenchymal transition [25]. This cell to cell communication confers a survival advantage and promotes the metastatic spread.

The prevalence of detected CTCs in the bloodstream, as expected, is higher in metastatic than in localized breast cancer [26, 27]. An important limitation to use CTCs in clinical practice is, at least as yet, the difficulty to detect them. In eBC, CTC prevalence increases with disease stage, ranging between 10% and 30% in different studies across all stages [28] (Table 9.1).

There are some methods to isolate CTCs by size or by identification of cell surface markers. The FDA-approved CellSearch[®] system [36–39] is a platform commonly used for the isolation and enrichment of CTCs in breast cancer to identify the presence of CTCs in bloodstream. This platform is based on the positive selection of CTCs by expression of the epithelial cell adhesion molecule (EpCAM) surface maker. However, as

Study	N	Stage	Method of detection	Year	Prevalence	
Muller et al. [29]	60	I–II	OncoQuick	2005	8%	
Pierga et al. [30]	118	I–III	CellSearch®	2008	20%	
Bidard et al. [31]	115	I–III	CellSearch®	2010	10%	
Molloy et al. [5]	733	I–III	CellSearch®	2011	7,9%	
Lucci et al. [32]	302	I–III	CellSearch®	2012	24%	
Rack et al. [33]	2026	IIb–III	CellSearch®	2014	21.4%	
Janni et al. [34]	3176	I–III	CellSearch®	2016	20.2%	
Bidard et al. [35]	2185	II–III	CellSearch®	2018	25%	

Table 9.1 Prevalence CTCs in selected clinical trials involved eBC

mentioned previously a mechanism that could (at least partially) explain the metastatic process and CTC dissemination is the phenotypic change of epithelial to mesenchymal and the loss of epithelial surface proteins. Therefore, we have to be aware that CellSearch® method excludes the CTCs that lack EpCAM, resulting in the underestimation of mesenchymal-like CTCs that have lost their epithelial features. Recently, a work in primary breast cancer published by Mego et al. revealed a different behavior of CTCs accordingly to the expression of epithelial or mesenchymal surface proteins [40]. This fact strongly suggests the necessity to incorporate new methods to identify the various subpopulation of CTCs from clinical samples. To overcome this limitation, some researchers describe new methods to detect CTCs independently of epithelial biomarkers, as using nucleases as CTCs biomarkers. Previous studies provided information about the elevated amplification of these enzymes in cancer patients regardless its mesenchymal or epithelial phenotype. Kruspe et al. described this novel method to detect these more aggressive cells, concluding that this approach was promising to examine CTC levels in early diagnosis [12].

One of the characteristics of CTCs as mentioned above, is their important heterogeneity, like the differences between epithelial versus mesenchymal phenotypes; but we also have to take into account the heterogeneity of the various breast cancer subtypes (based on hormone receptors and HER2 [41]). CTCs can be isolated and molecularly profiled to evaluate important clinical biomarkers to monitor disease and help guide therapy. In this setting, Riethdorf et al. assessed CTCs by CellSearch[®] in patients with nonmetastatic breast cancer enrolled into the GeparQuattro phase III neoadjuvant trial [42]. Two hundred and thirteen patients were included in the analysis, and 21% had CTCs before neoadjuvant treatment and 10.6% after neoadjuvant treatment. HER2-overexpressing CTCs were observed in 24.1% of CTCs positive patients and was restricted to ductal carcinoma and associated with high tumor stage.

In the same line, Ignatiadis et al. conducted a study with the aim to identify CTCs assessed by CellSeach® method, and HER2-positive CTCs in breast cancer patients [43]. According to experiments performed in cell lines, HER2-positive CTCs were defined by a population of CTCs with HER2 immunofluorescence intensity that was at least 2.5 times higher than the background. The study showed that 4.1% of patients with ductal/ lobular carcinoma in situ had at least 1 HER2positive CTC, 7.3% in eBC and 39.5% in metastatic breast cancer. No CTCs HER2 positive were detected in 42 women without breast cancer. In this line of research, Ligthart et al. in a prospective study evaluating HER2 CTCs in adjuvant and metastatic patients, defined CTCs HER2 positivity as overexpression in 3.5 times higher than the CD45 immunofluorescence intensity in 75% of CTCs in patients with \geq 5 CTCs. Using this cut-off, 9% of M1 patients that were HER2 negative had HER2-positive CTC status and conversely 29% with HER2 positive primary had negative HER2 CTCs [44, 45], showing the heterogeneity of tumor cells presents in blood circulation.

Krishnamurthy et al. evaluated HER2-positive CTCs by FISH from 88 patients with breast cancer stages I–IV [46]. Cells with a ratio of HER2:CEP17 > 2 in any CK+/CD45 or CK-/ CD45 cell was regarded as positive for HER2 gene amplification. CTCs were detected in 27.3% of patients and HER2-positive CTCs in 11.1%. Among patients with a HER2-negative primary tumor, 6.3% had CTCs-HER2. The overall rate of discordance in HER2 status was 15% between primary tumor and CTCs.

ER expression in CTCs has been less extensively studied, however in eBC, only approximated 25% of CTCs are ER positive, despite most primary tumors being ER positive. However, the lack of a validated assay for determining ER-positivity in CTCs and a lack of larger studies examining ER CTC expression limits the clinical utility of this finding [47].

Taken together, these findings suggest potential clinical implications for evaluating molecular markers in CTCs in breast cancer patients.

9.3 Prognostic Studies

The main body of evidence published related to CTCs is related to its capacity to provide prognostic information. Here we review the most relevant studies related to prognostic information in eBC according to the use of systemic chemotherapy or not. However, CTCs are not yet routinely used in clinical practice as a prognostic marker due to the lack of definitive studies showing clinical utility in terms of helping to safely select those patients who will benefit from adjuvant therapy.

9.3.1 Prognostic Studies of CTCs in Patients Who Did not Receive Adjuvant Chemotherapy

CTCs have been reported as an independent poor prognostic factor in eBC. European groups firstly showed the prognostic impact of disseminated tumor cells (DTC) in the bone marrow of breast cancer patients [48]; Molloy et al. evaluated CTCs and DTCs at primary surgery in 733 stage I or II breast cancer patients. CTCs were detected in 7.9% of patients, while DTCs were found in 11.7%. Both CTC and DTC positivity independently predicted poor outcomes: metastasis-free survival (MFS) and breast cancer-specific survival (BCSS) [5].

In 2012 Lucci et al. conducted a clinical trial with the aim of identifying CTCs by CellSearch[®] system and their association with prognosis in eBC. They prospectively collected blood samples in patients chemo-naive, with eBC. They found ≥ 1 CTCs in 24% of patients. The detection of

one or more CTCs identified a subset of patients with worse prognostic, both decreased progression-free survival and overall survival [32]. As it will be mentioned below, the prognostic significance of CTCs not only is qualitative but quantitative, so that patients with rising numbers of CTCs had poor outcomes.

9.3.2 Prognostic Studies of CTCs in Patients Who Received Chemotherapy for Early Breast Cancer

The study conducted by Rack et al. assayed CTCs by CellSearch[®] system in 2026 patients with eBC before adjuvant chemotherapy and in 1496 patients after adjuvant chemotherapy [33]. The rates of detection of CTCs were similar in patients receiving adjuvant or neoadjuvant chemotherapy (in order to 21.5–22%). The presence of CTCs was an independent poor prognostic factor and was associated with poor disease-free survival, poor distant disease-free survival (DFS), breast cancer-specific survival (BCSS), and overall survival (OS). The group of patients with at least five CTCs had significantly worse outcomes (DFS: HR = 4.51, 95% CI = 2.59 to 7.86; OS: HR = 3.60, 95% CI = 1.56 to 8.45). In this trial, the authors found that the patients with persisting CTCs before and after chemotherapy treatment had worse outcomes compared with the other subgroups in terms of DFS, and an important negative prognostic effect in the presence of CTCs previously systemic treatment [49].

In the neoadjuvant setting, Pierga et al. investigated the presence of CTC in pre and post neoadjuvant blood samples in 118 non-metastatic breast cancer patients [30, 50]. They found a significantly decreased DFS and OS in patients with \geq 1CTC. Similar findings in the neoadjuvant setting were found by Riethdorf et al. in patients enrolled in GeparQuattro trial [42]. The Beverly study, included 137 patients with inflammatory breast cancer (IBC) candidates to neoadjuvant treatment [51, 52]. The study analyzed the possible benefit of incorporated bevacizumab to standard chemotherapy and trastuzumab in the neoadjuvant scenario. Prior to neoadjuvant chemotherapy, 39% of patients had detectable CTCs. The detection of CTCs after four cycles of chemotherapy decreased from 39% to 9%. The authors found that the presence of CTCs at baseline was associated with shorter 3-year DFS (39% versus 70%, P < 0.01, HR 2.80) and shorter 3-year OS (P < 0.01) compared with the patients with undetected CTCs [52]. The pooled analysis including Beverly 1 and Beverly 2, suggests that the combination of pathological complete response (pCR) and CTCs detection could be a potential tool to identify a subgroup with better outcomes after neoadjuvant treatment: the subgroup of patients that achieved a pCR and undetected CTCs had an excellent OS (94% 3-year OS) [50]. The authors suggested that the prognosis of IBC relies on the achievement of pCR and highlighted the role of early hematogenous tumor dissemination as assessed by CTCs. Combining these two prognostic factors they reported a subgroup of IBC with excellent survival when treated with bevacizumab and trastuzumab-containing regimens.

9.3.3 Pooled Analysis of the Prognostic Value of CTCs in Early Breast Cancer

Janni et al. published a pooled analysis including 3173 patients with stage I–III breast cancer [34]. A total of 58% of patients included had nodal involvement and 42 had high-grade tumor. In this series, only 8.2% patients received neoadjuvant treatment and 79.9% received adjuvant treatment, including hormonal therapy and radiotherapy according to guidelines. The presence of CTCs was assessed by CellSearch[®] at time of primary diagnosis. The prevalence of CTCs was 20% and the presence of \geq 1 CTCs was an independent negative prognostic factor for DFS [HR, 1.82; 95% confidence interval (CI), 1.47–2.26], distant DFS (HR, 1.89; 95% CI, 1.49–2.40), BCSS (HR, 2.04; 95% CI, 1.52–2.75), and overall survival. The presence of CTCs was correlated with large size, high histological grade and nodal involvement. In a subgroup analysis, CTCs were not able to provide prognostic information in very low risk patients (T1 N0) and in hormone receptor negative, HER2 positive breast cancer subtype, probably by the small sample size in the last subgroup.

A Meta-analysis was published in 2018 by Bidard et al. Data from 2185 patients from EEUU, Japan and European countries were included. Blood samples from patients were collected before neoadjuvant treatment (n = 1574) and before surgery (n = 1200) and presence of CTCs was assessed by CellSearch® system. One or more CTC were detected in 25.2% of patients before neoadjuvant chemotherapy. The presence of CTCs was associated with tumor size. In concordance with previous studies mentioned above, the number of CTCs detected had a detrimental impact in OS, DFS and locorregional relapse-free interval, although not correlated with pCR [35]. The higher number of CTCs detected before neoadjuvant chemotherapy was associated with the HR of death.

9.4 Other Prognostic Studies

9.4.1 CTCs and Late Recurrences in Early Breast Cancer

Recently, a trial published by Sparano et al. provided evidence of an association of CTCs and late recurrence in hormone receptor positive HER2 negative breast cancer [53]. Analysis of CTCs were assessed by CellSearch[®] system in 547 patients without clinical evidence of recurrence between 4.5 and 7.5 years after primary treatment of stage II–III breast cancer. Only 5% of patients had detectable CTCs in blood circulation. They found a 12.5 risk-fold increased risk of recurrence in patients with CTCs compared to patients with undetected CTCs detected. An interesting finding was that the patients with CTCs were still receiving hormonal therapy and 4.4% had clinical recurrence, and these were predominantly in HR+ breast cancer. The detection of CTCs was observed 2.8 years prior to clinical recurrence. This provided for the first time evidence on the potential value of CTC detection during patient follow up and late clinical recurrence.

9.4.2 CTCs with EMT Phenotype and Prognosis

As mentioned above, circulating tumors cells are a heterogeneous population of cells including CTCs with partial or complete EMT phenotype. The prognostic value of CTCs has been demonstrated for epithelial CTCs. However, a subset of primary breast cancer patients shows EMT and stem cell characteristics [54]. EMT phenotype in breast cancer have been shown to be prognostically unfavorable, but the prognostic value of CTCs with EMT is poorly known in eBC and the currently used detection methods for CTC are not efficient to identify a subtype of CTC which underwent EMT. An interesting work published recently by Mego et al. identify a subset of CTCs with more aggressive behavior and patients with an inferior outcome in this setting [40]. In this work the authors evaluated the expression of EMT transcription factors (TWIST1, SANAIL1, SLUG and ZEB1) by PCR in real time. The patients with EMT-CTCs had inferior outcomes compared with patients without detectable CTC EMT. In this work the presence of CTC EMT was associated with p53 status and after a median of follow-up of 55 months, patients with CTC EMT in the peripheral blood had significantly poor DFS. This prognostic value was demonstrated in all subgroups and was most pronounced in the hormone receptor positive, HER2 negative subgroup independently of the adjuvant treatment administrated. Despite of the small sample size of the study, it provides for the first time evidence fo the prognostic value of CTCs with an EMT phenotype in eBC. The poor prognostic associated to EMT features of CTCs is in line with observations reported in primary breast cancer tissue. Along this line, Creighton et al. reported that the residual breast tumor tissue cell populations surviving after letrozole or docetaxel treatment were enriched for subpopulations of cells with both tumor-initiating and mesenchymal features, which may explain resistance to antihormonal and conventional chemotherapeutic drugs [55].

9.4.3 Dynamic Evolution of CTC During (Neo)adjuvant Treatment

Muller et al. analyzed patients with primary breast cancer at stage M0. They found that 8.3% of patients had CTCs after surgery and before initiation of adjuvant chemotherapy. During the course of adjuvant chemotherapy, repeated analysis of 20 M(0) patients revealed the occurrence of CTCs in 7 of 16 patients that were initially negative [29].

Pachmann et al. analyzed the presence of CTCs to monitor residual disease during adjuvant treatment with the aim to detect patients early who are at risk of relapse [56]. They analyzed serially the presence of CTC by epithelial surface markers in 91 non-metastatic primary breast cancer patients by an EpCAM-based laser scanning cytometric approach. Patients with initial reduction in CTC number followed by a significant increase (>10 fold compared with the nadir (lowest value) were the subgroup with the highest risk of subsequent relapse. Kwan et al. have developed a novel breast cancer CTCspecific assay, selecting 17 transcripts strongly expressed in breast derived tissue but absent in blood cells [57]. They tested its clinical utility monitoring response in high-risk breast cancer patients receiving neoadjuvant therapy. In 52 patients with localized breast cancer, the increase in a CTC-score after three cycles of neoadjuvant therapy was associated with residual disease at surgery. This study suggests a novel CTC assay to monitor response to neoadjuvant chemother-

Study	Туре	Status	Reference
Characterization of circulating tumor cells (CTCs) in patients with locally advanced or metastatic stage IV breast cancer	Observational	Recruiting	NCT01048918
Effect of Digoxin on Clusters of Circulating Tumor Cells (CTCs) in Breast Cancer Patients	Phase 1	(M1) Active	NCT03928210
Predictive Value of Circulating Tumor Cells in Neoadjuvant Chemotherapy Among Locally Advanced Breast Cancer Patients: a Single-center, Prospective, Exploratory Clinical Trial	Clinical Trial	Recruiting	NCT03732339
Circulating tumor cells (CTCs): a potential screening test for clinically undetectable breast carcinoma	Observational	Recruiting	NCT01322750
A pilot surveillance study to monitor natural killer cells and circulating tumor cells in women with previously treated non-metastatic triple negative breast cancer and women with previously treated non-metastatic breast cancer with a confirmed BRCA mutation	Observational	Active not recruiting	NCT02639832
Analysis of Circulating Epithelial Tumor Cells in Peripheral Blood in Patients With Primary Non-metastatic Breast Cancer Under Adjuvant Radiotherapy	Observational	Recruiting	NCT03935802

Table 9.2 Selected studies involving testing CTCs in eBC

apy. Further research is needed to further study its potential clinical implications.

9.5 Clinical Trials Based on CTCs

The presence of CTCs in eBC has been the bases for a few clinical trials (Table 9.2). Georgoulias et al. conducted a randomized phase II trial in patients with non-metastatic breast cancer with detectable CTCs before and after adjuvant chemotherapy based on an anthracycline regimen [58]. CK19 mRNA-positive CTCs were detected by RT-PCR and double stained CK(+)/HER2(+)cells by immunofluorescence. A total of 378 patients (310 HER2-negative and 68 with HER2positive eBC) were treated with adjuvant chemotherapy and 148 (39%) patients had detectable CK19 mRNA-positive CTCs before any adjuvant systemic treatment. The patients with persistence of CTCs (26%) at the end of adjuvant chemotherapy were randomized to receive trastuzumab or observation. Fifty-one (89%) of the 57 analyzed patients had HER2-expressing CTCs. In HER2negative breast cancer patients, after trastuzumab administration, 27 of 36 (75%) women became CK19 mRNA-negative compared to seven of 39 (17.9%) in the observation arm. In that study, the median DFS was significantly higher for the trastuzumab-treated patients. This result suggested that the administration of trastuzumab may eliminate chemotherapy-resistant CK19 mRNA positive CTCs and improve patient's outcome.

However, these results were not confirmed by a phase II trial conducted by Ignatiadis et al. that included 95 HER2 negative eBC patients with CTCs detected by CellSearch® after completing neoadjuvant chemotherapy and surgery [59]. These patients were randomized to receive trastuzumab or no treatment. The aim of the study was the eradication of CTCs at week 18 in the experimental arm. In 23.8% of the patients there was at least one HER2-positive CTC (6 patients in the trastuzumab arm and 9 in the observational arm). Fifty-eight patients were assessable for the primary end point, 29 in each arm. In 9 of the 58 patients, CTC(s) were still detected at week 18; 5 in the trastuzumab and 4 in the observation arm. The study was stopped by Independent Data Monitoring Committee recommendation for futility for the primary end point, concluding that the use of trastuzumab according to detection of CTC was of no benefit in terms of DFS or OS. Further research is needed in this area.

Related to radiotherapy, Goodman et al. recently published a potential relation between

CTC and benefit of radiotherapy in patients with eBC that undergo conserving surgery [60]. The study included patients enrolled in SUCCESS trial, and patients from NCDB. CTCs detection ranged from 19% to 24% and DFS and OS were related with the presence of CTCs. The patients that received radiotherapy after conservative surgery had longer local recurrence-free survival (LRFS), DFS and OS if at least 1 CTC was detected compared to patients that did not receive radiation therapy. In contrast, in patients with undetected CTCs, the addition of radiation therapy did not significantly improve patient outcomes. This clinical trial was the first study that incorporated CTCs as a predictive biomarker of radiation therapy in non-metastatic breast cancer and these results suggest CTCs as a potential tool to identify patients that potentially benefit of radiation. Based on these promising results, additional studies appear warranted.

There are additional registered ongoing studies of CTCs in eBC that will shed light on their potential utility in the upcoming years. A selection of these studies is presented in Table 9.2. Furthermore, CTCs may become a target per se in eBC, particularly CTC clusters, albeit current technology still does not allow their reliable detection in a sufficient proportion of eBC patients. This view is based on an interesting preclinical study suggesting the use of drugs that could segregate and prevent clusters formation in blood in metastatic setting [21].

9.6 Conclusions and Future Directions

There is definitive evidence on the prognostic role of CTCs in eBC but its clinical utility in daily practice is still lacking. However, the challenge is to develop biomarkers for prediction rather than prognosis. Clinical trials based on CTCs have provide promising, but not robust, responses to the potential use of CTC-guided therapy in eBC. Therefore further efforts should be directed at deepening the molecular characterization of CTCs and, importantly, to the design of clinical trials that could exploit the unique information that CTCs may reveal. It is also tempting to speculate that CTCs may also provide complementary information on the interplay of tumor cells with the immune system.

We have to take into consideration that the studies published to date mainly evaluated the presence of CTC based on the expression of epithelial surface markers. Future studies need to overcome this limitation and, hence, be based on methods that can detect CTCs with mesenchymal phenotype or detect CTCs based on their physical properties. There are also new methods to assess CTCs that can capture the heterogeneity of the tumor landscape and this may prove valuable in the future. In addition, the information of CTC enumeration and biological features should be combined with the emerging data provided by ctDNA. It is entirely reasonable to believe that the combination of CTCs and ctDNA testing may provide new ways to personalize in an unprecedented manner the management of patients with eBC.

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10

Clinical Relevance and Therapeutic Application of CTCs in Advanced Breast Cancer

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Abstract

Precision medicine through liquid biopsy represents an emerging approach in the management of cancer. The CTC count in blood samples from patients with advanced breast cancer is a powerful prognostic factor for both progression free and overall survival. Moreover, high levels of CTCs at any time during the treatment can reliably predict progression before imaging studies and/or tumor markers. Furthermore, there are works on the molecular characterization of the CTCs and their potential ability to guide the treatment in a dynamic way. However, their role remains controversial. Detection and enumeration of CTCs is variable among different tumors and is subjected to biases related mainly to their methodology, which is not completely standardized. In addition, they must demonstrate their clinical value to guide the treatment and a translation on patient's survival.

Keywords

Metastatic breast cancer · Circulating tumor cells (CTCs) · Prognostic value · Treatment monitoring · Precision oncology

10.1 Introduction

Advanced or metastatic breast cancer (MBC) is still an incurable disease, although the introduction of modern systemic therapies has improved prognosis. The current median overall survival time is approximately 2 years, varying from a few months to several years, depending on the molecular subtype and treatments received. As more knowledge is gathered regarding the specific molecular alterations of MBC, it becomes essential to define both prognostic (provide information on the evolution of the disease) and predictive factors (report on efficacy to a specific treatment). Likewise, techniques with the capacity to guide the treatment are needed, thus contributing to a better selection of specific therapies.

Detecting and isolating circulating tumor cells (CTCs) in the blood of patients with MBC is possible due to the development of very sensitive techniques. Although several commercially available methods exist for this detection, CellSearch[®] (Menarini Silicon Biosystems, Inc) is the only one approved in the United States for clinical use.

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Currently there are two main research lines related to CTCs in MBC. Firstly it was demonstrated that the CTC count before and during systemic treatment is a prognostic factor. This reflects the progression or response of disease to the treatment, so CTCs monitoring could help to identify earlier patients who do not benefit from therapy; however, an early change in treatment based on CTC count has not shown any survival benefit so far. Secondly, on-going clinical trials are looking into patient benefit from receiving targeted therapies based on the molecular profile of isolated CTCs. In this chapter we will revise these questions.

10.2 Prognostic Value of CTCs

10.2.1 Pivotal Study

The first study that confirmed the clinical applicability of CTCs in patients with MBC were published in 2004 [1]. Number of CTCs with a cut-off of 5 per 7.5 ml of blood (CellSearch[®]) was prognostic factor for progression-free survival (PFS) and overall survival (OS), regardless of other clinical factors. This was a prospective study that included 177 MBC patients with heterogeneous characteristics: all molecular subtypes, different metastatic locations, and pre-treated or not. Minimal follow-up was 38.5 weeks. In the group of 87 patients with basal \geq 5 CTCs/7.5 ml (49%), the median PFS and OS were 2.7 (95% CI 2.1-4.4) and 10.1 months (95% CI 6.3-14.6), respectively. In the 90 patients with <5 CTCs (51%), median PFS and OS were 7 (95% CI 5.8-8.9) and more than 18 months, respectively. They also observed that with <5 CTCs at baseline but \geq 5 CTCs at the first follow-up visit (n = 5), the results were similar to the poor prognosis group. In contrast, patients with high baseline scores in whom counts decreased below 5 at first follow-up visit (n = 33), had comparable results to the good prognosis group. Finally, in those patients with high baseline CTCs that decreased but not <5(n = 25), results did not correspond with the good prognosis group.

10.2.2 Other Studies

Although some studies have been published with inconclusive results, the vast majority of subsequent trials (detection ranges 31-61%), and at least two meta-analyses have validated the presence of ≥ 5 CTCs/7.5 ml as a negative independent prognostic factor in patients with MBC, as well as its value as a dynamic biomarker in different moments of the disease [2–17]. Some of these studies will be reviewed in a later section.

A meta-analysis published in 2012 confirmed this prognostic value of CTCs, both in early and advanced disease, at different times of treatment, and using different techniques: immunocytochemistry (CellSearch[®]) or RT-PCR ("real time polymerase chain reaction"), also suggesting the need to standardize the methodology. In this meta-analysis, both the HR for PFS (12 studies, HR 1.78) and OS (19 studies, HR 2.33) were stasignificant in MBC population tistically (n = 3065) [18]. In a subsequent meta-analysis with 24 studies in MBC patients (n = 3701), it was noted that CTCs are more frequently detected in primary HER2 + tumors with respect to other subtypes (RR = 0.73); and that high counts indicated worse responses to therapy (RR = 0.56), and poorer PFS (RR = 0.64) and OS (RR = 0.69) [19].

10.2.3 Clinical Value of the CTCs

Some studies have been published demonstrating CTC counts have more value than other clinical prognostic markers, such as plasma tumor markers [20]. Correlation between CTC count, radiological evaluation and patient survival has also been studied [21].

In 2014 a retrospective joint analysis from 1944 MBC patients who had participated in 20 studies in several European centers (EPAC Consortium) was published [22]. All patients had a baseline CTC count, prior to starting treatment. In addition, other clinical-pathological variables were collected, as well as new CTC counts. Based on these data, investigators developed a clinical prognostic model for PFS and OS and then assessed the added value of including CTC and serum marker levels to that model. At baseline 47% of patients had \geq 5 CTCs/ 7.5 ml. This group presented worse PFS (HR 1.92, 95% CI 1.73–2.14) and OS (HR 2.78, 95% CI 2.42–3.19) than <5 CTC/7.5 ml group (Fig. 10.1). Increase in CTC count reflected tumor burden, but it did not correlate with tumor subtype [22]. The increase in the CTC count 3-5 weeks after starting treatment was also associated with worse PFS (HR 1.85, 95% CI 1.48–2.32) and OS (HR 2.26, 95% CI 1.68–3.03) (Fig. 10.2). Finally, survival prediction improved when adding CTC count to the clinical-pathological models. Furthermore, prediction was even more accurate by adding changes in CTC count at 3-5 and at 6-8 weeks. On the other hand, adding CEA and CA 15-3 changes did not provide significant information. The conclusion is that initial CTC count, as well as early changes after treatment initiation, results in a strong and independent prognostic marker which adds value to the classic clinical variables. So, the authors propose to use prognostic information based on CTC counts to stratify patients within clinical trials, and to check prospectively if efficacy objectives (such as OS and PFS) are improved by CTCs monitorization [22].

Furthermore it has been suggested that the prognostic value of CTCs could vary according to MBC subtype. In a retrospective study with 517 patients, baseline CTC count showed prognostic value in all subtypes, more significant in hormone receptor positive (luminal) and triple negative, and less significant in HER2+ tumors, suggesting interaction between CTCs and treatments [23]. These results were reproduced in another retrospective study with 235 patients, confirming the prognostic value of CTC count in the global population and in patients treated with chemotherapy and endocrine therapy. In those treated with bevacizumab or anti-HER2 therapies, the negative prognostic value of baseline elevated CTC levels was lost, suggesting the therapeutic benefit of these drugs [12].

A recent combined analysis of individual data from patients with MBC from the 17 centers of the EPAC Consortium [22] plus a series from MD Anderson Cancer Center in Houston (n = 2436), was done. The authors propose evaluating the aggressiveness (prognosis) of the disease according to CTC count and classifying stage IV into two subgroups: IV-indolent and IV-aggressive. They consider the need to stratify



Fig. 10.1 Kaplan-Meier analysis of progression-free survival and overall survival, by baseline CTC count. (a) PFS. (b) OS. (Reproduced from [22])



Fig. 10.2 Kaplan-Meier analysis of progression-free survival and overall survival, by early change in CTC count. (a) PFS. (b) OS. (Reproduced from [22])

patients based on this classification, and then assessing the molecular and clinical factors to finally evaluate the true impact of treatments [24]. After CTC collection, 44% of patients were treated with chemotherapy; 37% with chemotherapy plus a biologic or targeted therapy; 13% with endocrine monotherapy; and the remaining 6% was classified as others. With a median follow-up of 15 months, there was a statistically significant difference in OS (36.3 vs 16.0 months, p < 0.0001) in patients with IV-indolent versus IV-aggressive stages (Fig. 10.3a). CTC count was also able to stratify patients with de novo disease (OS 41.4 vs 18.7 months, p < 0.0001). Likewise, OS was significantly better in IV-indolent regardless of prior treatments and disease location. According to the molecular subtypes, median OS was also significantly larger in IV-indolent versus IV-aggressive, both in hormone receptor (ER) positive (40.7 vs. 17.3 months) and in triple negative (23.8 vs 9.1 months), as well as in HER2+ (33.2 vs 19.4 months) (Fig. 10.3b-d). CTC count was the most significant predictor of all covariates (HR 2.71, 95% CI 2.35-3.12). Fig. 10.3e shows the forest plot for OS according to the different subgroups. To summarize, CTC count is useful to stratify patients with MBC, independently of tumor subtype, line of therapy and disease location.

Finally, the eighth edition of the "AJCC Cancer Staging Manual" recognizes a CTC count \geq 5/7.5 mL of plasma in patients with MBC as an unfavorable prognostic factor with a level of evidence II. This type of cancer is a pioneer in the incorporation of liquid biopsy findings to define patient risk groups [25]. However, CTC counts, as well as other molecular factors, have not been systematically included in TNM staging because their analysis is not implemented in most centers.

10.3 Characterization and CTCs Heterogeneity

The molecular characterization of CTCs could contribute to a better understanding of tumor biology and their mechanisms of metastatization



Fig. 10.3 OS stage IV-indolent versus IV-agressive, entire (a), ER+ (b), HER2+ (c), and triple negative (d) cohorts; and forest plot of OS according to subgroups (e). (Reproduced from [24])



Fig. 10.3 (continued)

and resistance. It could potentially contribute to the development of biomarkers and selection of targeted therapies [26]. The genomic profile of CTCs and primary tumors confirm a shared lineage, with some genetic divergence [27] consistent with the formation of metastasis as a result of a single clonal expansion [28]. It is known that the phenotypes and genotypes of the primary tumor, metastasis and CTCs often differ [29]. Ideally, therapeutic decisions should be based on the characteristics of the predominant disease at the time of relapse and at each progression. The characterization of CTCs in peripheral blood can be an alternative to tissue biopsy, as a less invasive and more dynamic test (repeatable, in real time). Hypothetically, CTCs represent the population of dominant tumor cells of a metastatic disease, so their expression profile could theoretically help us to predict the therapeutic response more accurately [30]. However, the identification and characterization of CTCs is not simple and requires extremely sensitive and specific techniques. CTCs represent a dynamic population that can originate in the primary tumor as well as in the metastasis or in both, and its characterization provides us with information, whose clinical utility is yet to be determined [31].

There are studies that show the possibility of molecularly characterizing CTCs and their prognostic correlation, but it has not yet been proved that this can render a prediction to the corresponding targeted therapy response. Research with cell lines derived from CTCs of MBC ER+ patients made it possible to determine sensitivity to new drugs directed against potentially treatable targets [32]. Gene expression studies with a so-called metastasis-initiating cells phenotype have also been published, reporting the induction of metastasis in xenograft assays [33]. But the greatest development on this field is aimed at characterizing biomarkers in CTCs with clinical implications, or gene expression profiles associated with the proliferation and acquisition of mesenchymal or stem cell phenotypes [34–36]. EMT shares some stem-cell properties, including resistance to conventional therapies. More than half of CTCs of patients with MBC show EMT and stem markers, whose presence correlates

with a genotype more resistant to drugs [37] and with few responses to conventional treatments [38]. These markers may represent a potential therapeutic goal.

HER2 positivity in CTCs ranges between 27–63%. So, CTCs/HER2+ are frequently detected in tumors (tissue) HER2+; but it has also been described primary (tissue) tumor HER2and CTCs +, and vice versa, in percentages between 49 and 77% [39–44]. In a retrospective study the correlation between CTCs and primary was 69%, and with metastasis 74%. It was also observed that the CTC/HER2 + patients presented a PFS significantly longer than the CTC/ HER2-, although with no impact on OS [45]. Conversely, Hayashi et al. observed that patients with CTC/HER2 + had a shorter PFS and OS [46]. Another study in which frequent discordance was found, it did not observe a prognostic impact [47]. We do not know if these discrepancies could be due to the administration of different therapies.

The expression of ER in CTC has been less studied. Despite being by far the most frequent phenotype, in early disease only approximately 25% of CTCs are ER+ [48, 49]. Unlike the expression of HER2, which in CTCs seems to be lost or gained with a similar frequency, ER expression is more frequently lost in the evolution from primary to CTC [50]. However, the lack of a unified methodology to determine ER+ in CTCs and the absence of extensive studies limits the value of these findings. Recently, a group has developed the so-called CTC-Endocrine therapy index (CTC-ETI), a score based on CTCs enumeration and characterization of ER, Bcl-2, HER2 and Ki67 using CellSearch®. A high CTC-ETI index was attributed to patients with high CTC counts and with low expression of ER and Bcl-2 and high levels of HER2 and Ki67 [51]. The Phase II COMETI trial (NCT01701050) is evaluating the value of the CTC-ETI score to identify women with refractory endocrine MBC.

In another study, CTCs are characterized by the presence of mutations in PIK3CA, in addition to HER2 expression, as a biomarker for inhibitory drugs already available for clinical use (Alpelisib). Of 290 patients included, PIK3CA mutations were analyzed in 33 patients with >5 CTC/7.5 ml, with great heterogeneity in mutations of PIK3CA and HER2 expression.

Therefore, clinically relevant genomic aberrations such as those of PIK3CA are detectable in a single CTC [52]. Other studies have been published regarding the characterization of the PIK3CA status in CTCs of MBC [53]. Another interesting question is the determination of PD-L1 in CTCs. Immunotherapy (Atezolizumab) has already shown benefit in triple negative MBC PD-L1+. One study shows expression of PD-L1 of CTCs in 11 of 16 cases (68.8%) MBC ER +/ HER2- patients [54].

Rossi et al. evaluated the usefulness of combining CTCs and circulating tumor DNA (ctDNA) as a prognostic prediction in MBC. Retrospectively, in 91 patients, CTCs were found in 85%, with mutations in 84% of the samples. The genes most frequently mutated were TP53 (52%), PIK3CA (40%) and ERBB2 (20%). A statistically significant difference was observed in PFS and OS for patients with values < 5 CTCs versus ≥ 5 or more; and in percent of ctDNA <0.5% versus ≥ 0.5 ; and having a number of genetic alterations <2 versus \geq 2. They conclude that liquid biopsy can be used as an effective prognostic tool and that the characterization of CTCs is viable [55]. It has also been pointed out that epigenetic silencing in the promoter regions of tumor suppressor genes can be confirmed in CTCs of MBC [56].

10.4 CTCs and Monitoring Treatment in Advanced Breast Cancer

The isolation and quantification of CTCs in early or locally advanced [40, 57–61] or MBC has shown independent prognostic value in several clinical trials and meta-analyses [1, 18, 19, 22]. In addition, changes in CTC counts can reflect early the efficacy of treatment and allows the monitoring of the disease [62]. As an example, in a recent study in patients with stage III-IV breast cancer, differences were observed in CTC responses after treatment according to age groups. The authors propose a combination of baseline CTCs along with age as a new potential criterion for treatment selection [63].

10.4.1 Can Changes in the Quantification of CTCs Be Useful as Early Predictor of Treatment Efficacy?

A multicenter study with 177 MBC patients, in addition to others with advanced colorectal and prostate cancer, was done. The prognostic influence of changes in CTC counts during treatment was studied. In all three tumors, persistence of high CTC counts was related with worse OS, suggesting that treatment was not being effective; unlike those patients who showed a decrease below the unfavorable chosen cut-off (\geq 5 CTC/7.5 ml for MBC), in whom the prognosis improved [64].

The first study in MBC showing the usefulness of monitoring CTCs as a predictor of response is performed in 68 patients treated with chemo- or hormone therapy. In addition to standard radiological evaluations every 3 months, CTCs were quantified at the beginning of treatment and with each cycle for the first 6 months, and then with each radiological evaluation. A strong correlation was demonstrated between CTC monitoring and radiological progression of the disease. Moreover, changes in CTC counts suggested progression weeks before radiologic evaluation. The authors conclude that serial determination of CTCs can identify treatment efficacy earlier than the standard evaluation [15]. In addition, we have previously mentioned results by Bidard et al., which also demonstrated the clinical value of CTC monitoring in MBC, as well as the limited validity of serum tumor markers [22]. Likewise, in another study in 117 patients with MBC, CTC counts were taken at baseline, before the first cycle of chemotherapy and before the second. Patients with <5 CTC on day 21 had significantly better clinical benefit rate (77 vs 44%), PFS (9.4 vs 3 months) and OS (38.5 vs 8.7 months) versus those with \geq 5 CTCs [16]. Other authors report similar results [13, 21].

Finally, a recent meta-analysis including 50 studies with 6712 patients with early and advanced breast cancer confirms CTC levels as predictors of response to treatment [65]. Therefore, it can be concluded that changes in the quantification of CTCs during treatment are predictors of efficacy earlier than standard radiological assessment.

10.4.2 Do Decisions Guided by the Use of CTCs Have an Impact on Treatment Efficacy Outcomes?

Before generalizing its routine clinical use, it must be demonstrated that patients with persistently elevated CTCs during systemic treatment benefit from early change of the therapeutic regimen, in efficacy parameters (PFS, OS), safety (avoiding toxic therapies) and/or in cost reduction (avoiding inefficient and expensive treatments and procedures). Phase III prospective interventional clinical trials investigating these issues in MBC have been designed [66].

In the SWOG 0500 study (NCT00382018), patients in first line of chemotherapy with baseline count \geq 5 CTCs/7.5 ml, who maintained high levels (\geq 5) after the first cycle of treatment, were randomized to continue the same regimen (until radiological or clinical progression) or to change early to a second line. Between 2006 and 2012, 624 patients were screened, of which 288 were randomized. No differences were observed in OS or PFS between the treatment arms: 10.7 vs 12.5 and 3.5 vs 4.6 months, respectively. Investigators conclude that this situation indicates chemoresistance, and the lack of an effective alternative therapy could explain the absence of impact on the outcomes [17].

The French trial CirCe01 (NCT01349842) includes 304 patients in the third line of chemotherapy with CTC levels \geq 5/7.5 ml that are randomized to standard management based on clinical-radiological evaluation or based on CTC dynamics. The primary endpoint is OS benefit, with other secondary endpoints including economic analysis. The results of this trial are not available. Another French study, the STIC-CTC METABREAST (NCT01710605), planned a recruitment of 994 patients with endocrinedependent MBC, where the choice of first-line treatment is based on the levels of CTCs: endocrine therapy for a count of <5 CTCs/7.5 ml or chemotherapy for \geq 5. The results are also not available.

These and other smaller similar studies (COMETI/NCT01701050, CTC-EMT/ NCT02025413, PRO OncAssay/NCT01048099, Trastuzumab & Vinorelbine/NCT 01185509) try to demonstrate that the persistence of elevated CTC levels during treatment indicates early ineffectiveness and that patients would benefit from an early change to another effective treatment (if any). On the other hand, toxicities and unnecessary risks for patients and extra costs for the system would be avoided.

10.5 Precision Oncology and CTCs in Advanced Breast Cancer

Previously reviewed approaches would reach their maximum clinical utility used as a dynamic treatment guide, according to the molecular alterations found in the CTCs, and showing a favorable clinical impact for the patient. This should be the ideal expression of the precision oncology. In this sense, a review and classification of genomic alterations of breast cancer according to their level of evidence for actionability has been published, following a scale developed by the European Society of Medical Oncology (ESMO), the ESMO Scale for Clinical Actionability of Molecular Targets (ESCAT). Large databases analyzed suggested around 40 recurrent driver alterations. Clinical trials were reviewed following various sources to evaluate the efficacy of drugs matched to these genomic alterations. The targetability for most studied alterations was graded according to the ESCAT scale, which classifies the molecular target at different levels (I–V and X) according to the available evidence. An important limitation of this classification is that it focuses on DNA alterations. In level I, alteration-drug match is associated with benefit

in clinical trials, so access to treatment should be considered standard. In level II, it is considered that there is evidence of activity with drugs associated with the alteration, but without information on the magnitude of the benefit due to a lack of prospective data. In this way, amplification of ERBB2, germline mutations of BRCA1/2 and PIK3CA mutations were classified at level of evidence IA. NTRK fusions and microsatellite instability (MSI) were classified as IC. Mutations in ESR1 and loss of PTEN were classified in level IIA; and mutations in ERBB2 and AKT1 in level IIB [67].

10.5.1 Comparison of Primary Tumor Molecular Profile Versus CTCs

The knowledge of the correlation between molecular alterations of CTCs and the primary tumor is essential to support precision oncology. In one study with 62 MBC patients expression levels of 35 genes were studied; and in 48% the profile was discrepant between CTCs and primary tumor. In 24% ER was different, and patients with primary ER- and CTCs/ER+ presented a significant median time to treatment longer (8.5 versus 2.1 months). It is concluded that differences in the ER status could have therapeutic and prognostic implications [68]. Another study showed that some CTCs from patients with tumors originally ER+/HER2- could acquire a HER2+ phenotype and/or activation of different signaling pathways under therapeutic pressure. The coexistence or conversion between these states could make it easier for tumor cells to overcome stressors [69]. However, a phase II study failed to prove benefit with Lapatinib as a single agent in patients with HER2- initial tumors and CTCs/HER2+ [70].

10.5.2 Circulating Stem Cells

It is considered that circulating stem cells represents a particularly aggressive, invasive and proliferative subgroup of MBC, which makes them a target of great value [71]. In vivo xenograft models anti-CD44 antibodies (stem-cell marker) reduced tumor growth [72]. They are currently being investigated inhibitor tirosine-kinase drugs for PAR6A, Notch1, Hedgehog, Wnt, integrins, claudins, and Rho GTPases, all of them signaling pathways activated in stem cells or involved in the regulation of EMT [73].

10.5.3 CTCs, ESR1 and TK1: Importance in the Endocrine Treatment

One of the most studied mechanisms of endocrine acquired resistance is the appearance of specific mutations in the ESR1 gene. It has been related to lower response and resistance to aromatase inhibitors. One study evaluated ESR1 mutations in CellSearch[®]-enriched CTCs of patients with MBC on endocrine treatment. In cohort 1 were included patients in first line endocrine treatment (n = 43), and in cohort 2 patients progressing in any line of endocrine therapy (n = 40). In a subgroup of them, the mutation status of ESR1 in CTCs and paired cfDNA of each patient was compared. They observed that the mutation of ESR1 in the CTCs was not enriched in cohort 2 (8%) compared to the reference cohort (5%). Instead, in the cfDNA the ESR1 mutation was enriched in cohort 2 (42%) compared to the reference cohort (11%). Therefore, the sensitivity to detect mutations in cfDNA was higher than in the fractions enriched with CTCs. In addition, they concluded that ESR1 mutations are essential in the endocrine treatment resistance [74]. Another work studied the ESR1 methylation in tissue, CTCs and ctDNA of paired plasma in patients with MBC ER+ treated with Everolimus and Exemestane, combination usually used in second or third line. Methylation was detected in 38.5%, 23.3% and 7.4% of tissue samples, CTCs and ctDNA, respectively. Also, correlation was observed between methylated ESR1 and lack of treatment response [75].

Finally, one study analyzed the role of thymidine kinase-1 (TK1, proliferation marker) in blood, CTC counts and mutations of ESR1 and PIK3CA in ctDNA of patients with MBC ER+/ HER2-, and the correlation with the benefit of endocrine therapy. A high level of baseline TK1 activity and a high CTC count were observed in the cases with worse PFS rates, as well as a lower response to endocrine treatment. The study concluded that the analysis of TK1 activity together with the CTC count can be considered as a possible prognostic, predictive and monitoring marker for endocrine therapy [76].

10.5.4 Choice of Treatment According to CTCs in Advanced Breast Cancer

Beyond the studies that evaluate the clinical utility of enumeration and characterization of a limited number of markers, CTCs could be a source of tissue for molecular screening. Several groups have demonstrated the feasibility of analyzing enriched fractions or pure CTCs and study the expression of a series of preselected transcripts, what has revealed a wide heterogeneity of CTCs at the transcriptional level [77, 78]. In addition, efforts are directed to identify mutational profiles of CTCs in various types of cancer. We also have evidence that they can be used as a tissue source for drug sensitivity testing. In fact, the ex vivo culture of CTCs allowed the identification of mutations in ESR1 in three of six cell lines derived from CTCs from patients with MBC ER+ pre-treated with aromatase inhibitors [32]. These mutations are very rarely observed in primary tumors or without previous treatment. Using these cell lines derived from CTCs, these mutations were confirmed as conferring resistance to Tamoxifen, Raloxifene and Fulvestrant, and sensitiveness to Raloxifene or Fulvestrant combination with an HSP90 inhibitor [32].

Whether we can choose and/or guide the systemic treatment in patients with MBC according to CTCs and its phenotype is the objective of several interventional on-going trials, whose results are pending. The DETECT trials investigate the therapeutic selection according to levels of CTCs and/or their phenotype. The accompanying translational research of all of them attempt to generate additional knowledge [79]. There are three studies depending on the MBC subtype: DETECT III, DETECT IV and DETECT V. In the first two trials, presence of CTCs is mandatory for inclusion and changes in their levels during treatment are evaluated by several blood samples. DETECT III (NCT01619111) includes patients with HER2- tumors and at least one positive CTC for HER2, randomized to receive standard systemic treatment at the physician's choice versus +/-Lapatinib. Patients with MBC HER2- and CTCs HER2- were included in DETECT IV trial (NCT02035813), receiving endocrine therapy plus Everolimus in ER+ tumors, or Eribulin (cytotoxic) if ER+ with clinical indication of chemotherapy or triple negatives tumors. In DETECT V study (NCT02344472), HER2+ tumors are included and treated with dual targeted therapy (Pertuzumab/Trastuzumab) in combination with chemotherapy or endocrine therapy (based on their expression of hormone receptors).

10.6 Discussion and Comments

Precision medicine through liquid biopsy represents an emerging and unstoppable approach in the management of cancer, which considers the intra- and inter-tumoral genetic variability, and which is transforming biomedical research [80]. In Spain, a proposal for a national strategy has been developed to regulate its implementation, guaranteeing technical quality and equitable access to its use, while also safeguarding the sustainability of the national health system [81]. Beyond their enumeration, CTC technologies advance towards the use of these cells as an accessible and valid source for dynamic analysis of the tumor. It is particularly interesting to know, as soon as possible and for each progression, the probability of response to treatment as well as the identification of resistances. However, detection and enumeration of CTCs is very variable among different tumors and is subject to biases related mainly to their detection methodology, which is not completely standardized. In addition, they must demonstrate their value to guide the treatment with clinical translation on patient's survival.

The CellSearch® platform is the only one licensed by the FDA for the isolation of CTCs and their prognostic enumeration in breast, colorectal and prostate cancer [64]. We have not yet reached the maximum benefit that CTCs can offer, and more evidence from prospective studies is needed for its use in another settings. In this way, they must prove to be a representative and relevant sample of the tumor biology versus the tissue samples or other liquid biopsy techniques (ctDNA, ctRNA, exosomes...). Regarding tissue biopsy, CTCs have the advantage of the accessibility of blood or other fluids, which implies the possibility of carrying out samples repeatedly in a non-invasive manner, providing us with realtime information on tumor variability [82].

The detection and measurement of free or tumor DNA (cf/ctDNA) as a biomarker has been widely developed. Dawson et al. evaluated their value in monitoring treatment response in MBC, comparing ctDNA with CA 15-3 and CTCs in 30 patients who received chemotherapy. Both ctDNA and CTC count were associated with worse prognosis, while CA 15-3 levels did not [83]. In a retrospective study from 117 patients with MBC also was reported that CTCs and ctDNA presented a similar prognostic value [84]. The analysis of ctDNA is attractive because the plasma can be easily extracted and analyzed without the prior need to isolate and enrich a small population of cells, and it is possible to identify it in the absence of detectable CTCs. For this reason it is likely that ctDNA analysis is the preferred option for genotyping and monitoring the response to treatment [85]. However, both provide techniques can complementary information.

The analysis of CTCs provides the opportunity to study the entire cell, with its morphological assessment, also providing DNA, RNA, proteins, and the opportunity to perform ex vivo functional studies and cultures. An important limitation of CTCs is that they may not fully reflect the biology of the underlying tumor [86]. In addition, there are several phenotypes within them, epithelial, epithelial-mesenchymal, mesenchymal, and stem-like [87]. However, the standard CellSearch[®] platform uses epithelial markers expression and excludes those of epithelialmesenchyme transition [36] and stemness, so that these phenotypes may not be detected. Finally, it is possible that CTCs do not reflect exactly intertumoral heterogeneity but they detect a specific subpopulation [78]. For all of this, some panels of experts have concluded that the CTCs should not be used to influence treatment decisions in MBC at this time [88, 89]. It is necessary to know results of prospective and randomized clinical trials that allow us to confirm the validity of CTCs monitoring and especially the clinical impact of an early change of molecularly and dynamically guided treatment. In addition, according to the hypothesis of the aforementioned study compiled by Cristofanilli et al. despite the significant benefit of the drugs, the joint inclusion in the studies of indolent and aggressive disease can negatively impact the final results. Their findings suggest that clinical and molecular variables are insufficient to adequately stratify patients and that this heterogeneity can be reduced by considering their two subgroups of stage IV, as a step towards a more individualized approach [24].

Recently the presence of clusters of CTCs has been valued. In preclinical models, their oligoclonal nature increases up to fifty times the ability to develop distant metastases against isolated CTCs [90, 91]. It is suggested that these clusters with subclonal alteration profiles can initiate mechanisms of oncogenic cooperation and that their analysis can be highly informative of the biology of the tumor. On the other hand, once isolated CTCs ex vivo, it is possible to expand them in cell lines or in immunocompromised murine models and establish xenograft models (CDX), with molecular profiles identical to those of origin [33, 92].

It has been extensively confirmed that high levels of CTCs at any time during MBC treatment are associated with tumor progression and can reliably predict it before imaging studies and/ or classic tumor markers, pointing out resistance earlier. In addition, as we have also indicated, there are works on the molecular characterization of the CTCs and on their potential ability to direct and individualize the treatment in a dynamic way. However until now, the role of CTCs and liquid biopsy techniques in the follow-up of patients with advanced disease is controversial, partly due to the absence of powerful predictive biomarkers and effective treatments.

Different studies suggest that use of molecularly directed agents outside their indications does not necessarily improve outcomes versus standard care in patients with pre-treated MBC. The SAFIR01 trial aimed to define the proportion of patients in whom targeted therapy could be offered based on the results of the genomic analyses. A total of 423 patients with MBC were included; however, only 55 patients (13%) received targeted therapy on a genomic basis. Of these, only 4 had an objective response and 9 showed no evidence of disease progression for ≥ 16 weeks, assuming a clinical benefit in 13 of 55 patients (23.6%) [93]. Similarly, in the randomized phase II trial SHIVA, of 741 patients with solid tumors pre-treated and refractory to standard therapies, 293 had a specific molecular alteration and 195 (40 with MBC) were assigned to receive an agent directed by molecular alteration or to standard treatment. It was stratified based on three signaling pathways: hormonal PI3K/AKT/mTOR receptor, and RAF/ MEK. Among treated patients, median PFS was 2.3 months with targeted therapy versus 2 months in control group [94]. By contrast, a large metaanalysis of 570 phase II studies (32149 patients) showed better results with a personalized versus a non-personalized approach, with significant higher responses ratio (31% vs 10.5%), median PFS and OS (5.9 vs 2.7 and 13.7 vs 8.9 months, respectively) [95]. Together, these data suggest that it is possible to identify genomic alterations in MBC and in other tumors. However, a greater evaluation is necessary on the predictive capacity of these findings; and more important, to have proven drugs against this molecular alterations, before using them in daily clinical practice.

The new NGS and ddPCR technologies have a good analytical validity, but more work is needed to establish their usefulness and the added clinical value of the expansion from individual genetic tests to large genetic panels. Experts agree that we need standardized bioinformatic methods for the interpretation of genomic data and that trials in precision medicine should be stratified according to the level of evidence available for the genomic alterations identified [96]. Thus, in breast cancer five potent markers can currently be used to indicate treatment: expression of the estrogen receptor, progesterone receptor (PgR), Her2 proteins (ERBB2), BRCA mutations, mutations in PIK3CA and expression of PD-L1. According to some authors, an optimal panel for breast cancer clinical trials could add mutations of AKT1, PTEN, ESR1, KRAS, BRAF, NF1, other HRD genes (RAD, ATM, ATR), and amplifications of NOTCH3, CCND1, CDK4, Rb, IGFR1 or FGFR1 [97].

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