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Tumor Liquid Biopsies

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Circulating Tumor Cells (CTC)

Pathophysiology of Tumor Cell Release into the Circulation and Characterization of CTC

Tilman Todenhöfer, Klaus Pantel, Arnulf Stenzl and Stefan Werner

The traditional model of metastatic progression postulates that the ability to form distant metastases is driven by random mutations in cells of the primary tumor. These mutations remain rare until clonally expanded and selected at secondary metastatic sites (Fidler and Kripke 1977). More recent models, however, propose that metastasis is an extension of primary tumor progression, not a distinct step with characteristic mutational determinants (Vanharanta and Massague 2013). Still, clonal heterogeneity within primary tumors is a source for the selection of metastatic cancer cells, and circulating tumor cells (CTCs) originating from the primary or secondary tumor lesion bear in themselves important information on the molecular characteristics relevant to tumor progression and cancer therapy. Thus, the concept of liquid biopsy for the analysis of CTC in the blood of cancer patients is of particular importance because these cells are biomarkers with a biological function in metastatic development, and their analyses might reveal important key mechanisms of cancer progression and cancer therapy (Alix-Panabieres and Pantel 2016). Metastatic progression is a biological cascade with an abundant dynamic and kinetic diversity across different cancer types. The metastatic cascade consists of several stochastic events comprising active cell migration, local invasion, intravasation of tumor cells into blood vessels, dissemination, arrest at secondary and primary sites, extravasation at distant sites, colonization, engraftment at distant sites, and finally formation of clinically detectable metastasis (Vanharanta and Massague 2013). Mainly, epithelial-to-mesenchymal transition (EMT) has been discovered as important process for dissemination and the tumor cell release into the blood stream. Beyond that tumor cells with stem-cell-like characteristics have

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also been postulated as important drivers of tumor progression and colonization at distant sites. Because features of both EMT and stemness are considered to be of particular functional relevance for metastatic progression, different markers for CTC analysis have been proposed to assess these characteristics (Werner et al. 2017). The corresponding characterization of CTC has the potential not only to yield important insights into molecular changes of advanced tumors but also to facilitate risk prediction (Alix-Panabieres and Pantel 2016).

1 Role of Epithelial-to-Mesenchymal Transition for Tumor Cell Release

Different studies with preclinical models have shown that EMT is a central process in the progression of solid cancers (De Craene and Berx 2013). Tumor cell dissemination from the primary lesion includes cell invasion into blood vessels and requires a phenotypic change of cell differentiation comprising augmented motility and changes of the cytoskeleton (Thiery 2002). These changes allow tumor cells to invade the walls of small vessels and to enter the blood circulation. This process represents a first but essential step of tumor cell dissemination and metastatic progression (Joosse et al. 2015; Schilling et al. 2012). Once they have entered into the blood stream, tumor cells are able to travel to distant organs, like the bone marrow (Mohme et al. 2017; Todenhofer et al. 2015). After extravasation at distant sites, the tumor cells can directly form metastases or switch into a latent state called tumor cell dormancy, which is thought to be the basis for delayed metastatic outgrowth (Uhr and Pantel 2011). The molecular mechanisms underlying tumor dormancy and development of metastases have not been fully elucidated yet but and are under intense investigation. Nevertheless, therapeutic intervention at this point holds great potential to inhibit tumor cell progression and to treat patients more effective (Uhr and Pantel 2011). At the onset of tumor cell dissemination, environmental conditions as well as several proteins have been identified that significantly contribute to EMT in cells from primary solid tumors. These environmental conditions comprise physical and mechanical stress, including hypoxia and radiation (Cannito et al. 2008). Under hypoxic conditions, tumor cells upregulate the transcription factor hypoxia-inducible factor 1 alpha (HIF1α) which represents an important protein in the cell response to low oxygen levels (Ye et al. 2016). HIF1α upregulates the expression of various genes including genes involved in EMT, like N-cadherin and vimentin. Triggering of EMT by hypoxia is supposed to give rise to resistance of tumor cells to therapy (Marie-Egyptienne et al. 2013). Interestingly, mechanical stress to tumor cells such, as needle biopsies, has also potential to induce EMT as shown by upregulation of EMT marker, which could contribute to the process of tumor cell dissemination (Mathenge et al. 2014). Nevertheless, the biological role of EMT in CTC is still inadequately understood. Up to the present time, evidence is lagging that really all tumor cells that disseminate from the primary tumor mass have to undergo EMT during the invasion of blood vessels or their passage through the

blood circulation. Furthermore, no preclinical model could convincingly verify that EMT is inevitable for epithelial tumor cells to start dissemination and to become a CTC. Moreover, the clinical relevance of mesenchymal characteristics of CTC is unclear, although recent evidence suggests that the occurrence of CTC with mesenchymal characteristics is related to aggressive disease (Krawczyk et al. 2014). The biological process of EMT is closely related to the detection stem cell characteristics (Barriere et al. 2014). Cancer stem cells are a distinct subpopulation of tumors cells having the potential of self-renewal and proliferation and are therefore expected to be the main promoter of tumor growth (Pardal et al. 2003). The concepts of specifically targeting stem-like tumor cells have become an important goal in treatment for cancer (Yoshida and Saya 2016). This concept further implicates that tumor stem cells with the infinite potential for renewal and replication form the only subpopulation of CTC that is competent to form distant metastases (Kreso and Dick 2014). Consequently, identifying CTC with stem-cell-like traits is assumed to be highly relevant when interpreting the clinical impact of CTC (Yang et al. 2015). To date, the exact molecular features of these cells are still not entirely understood. It is necessary to develop new technological platforms for enrichment and molecular analysis of stem-like CTC. These platforms should include in vitro assays and culture of CTC to evaluate the key characteristics of tumor stem cells. Up to now, most utilized analytical assay does not allow to assess replication and self-renewal ergo culture of CTC, limiting the evaluation of the main features of stem cells (Alix-Panabieres et al. 2016).

2 Role of Cancer Stem Cells in Metastatic Progression

As already mentioned above, CTC characteristics of both EMT and stemness are closely related and considered to be of major importance for metastatic progression. In benign tissues, stem cells are a rare, slow-proliferating cell type with special biological characteristics. In this connection, their capabilities to self-renew and to differentiate into different type of cells are of particular importance. Generally, these features allow stem cells to eventually undergo extensive proliferation while preserving an undifferentiated, plastic cell state that helps to sustain tissue homeostasis (Clevers 2011). The traditional multistep model of malignant transformation and tumor progression likewise hypothesizes a single long-lived founder cell for cancer formation, in which accumulation of fundamental genetic alterations drives malignant transformation (Fearon and Vogelstein 1990). The central point of the cancer stem cell (CSC) model is that tumors might be driven only by a subgroup of tumor cells with stem-cell-like properties. In other words, solid tumors might consist of cells that are functionally heterogeneous, with only a subgroups of cells being liable for maintenance and progression of the tumor (Clevers 2011). Because cells from solid tumors that express mesenchymal markers are often also classified as CSCs, it has been proposed that during tumor progression, the highly relevant stem-cell-like phenotype can be acquired through EMT, in particular characteristics

that are related to metastatic progression, e.g., increased invasiveness and locomotion, are also CSC features (Singh and Settleman 2010). Hence, metastatic cancer cells, which have probably acquired epithelial-to-mesenchymal plasticity, might possess a CSC phenotype. The CSC concept raises important implications regarding detection and classification of CSCs as the biological and therapeutic relevant subgroup of CTC. First, the CSC model implies important consequences for assessment of severe clinical problems, like therapeutic resistance and tumor recurrence. Chemo- and radiotherapy are widely used to treat cancer patients of different cancer entities. These therapeutic approaches predominantly aim to remove rapidly cycling and proliferative cells. On the other hand, it is thought that CSC as potential key players of tumor formation and metastatic progression have a low proliferative activity. Consequently, CSCs may generally be capable to persist these treatments and start cycling after chemotherapy withdrawal to enable tumor recurrence in a clinical setting (Mitra et al. 2015). In this context, the CSC model explains convincingly that rare non-cycling tumor cells might cause tumor recurrence after therapy, which is the almost-inevitable treatment outcome of solid tumors. For that reason, detection and classification of potential CSC subsets in the whole CTC population may hold useful information about treatment efficacy and occurrence of therapy resistance. This could enable better-informed approaches to treat cancer patient more effective. A second implication for CSC model points to the role of stem-like cells in metastasis formation. Metastatic outgrowth at a distant site is the foremost cause of cancer-related death of solid tumors. Single tumor cells that have been released from the primary lesion are the latent new seeds of a secondary lesion at the site of metastasis (Braun et al. 2005; Werner and Pantel 2017). Conversely, metastatic progression is a very ineffective process, and the detection of CTC in blood samples does not certainly indicate that the patient is suffering secondary metastases. However, simple CTC count is a good predictor of relapse in patients with solid tumors like breast cancer (Rack et al. 2014). This suggests that detection of CTC in patient blood is indeed associated with clinical manifestation of micrometastases or at least with disseminated tumor cell in secondary organs. The capability of metastatic outgrowth is, however, a bottleneck in cancer progression. Accordingly, only a minority tumor cells present the whole CTC population and are capable to successfully form overt metastasis in a distant site at all (Coumans et al. 2013). For that reason, it has been postulated that metastases arise from a rare subset of CTC, which may bear stem-cell-like characteristics. These so-called metastasis-initiating cells (MIC) are considered to be the most dangerous subgroup in the entire CTC population, with self-renewal, multipotency, and metastasis-initiating competences (Celia-Terrassa and Kang 2016). Hence, detection and subsequent characterization of putative MICs in the whole CTC population from patient-derived blood samples could help to predict which patients are most likely to suffer distant metastasis and also to allow the analysis of biology behind metastasis formation. Finally, tumor dormancy is another unwieldy clinical phenomenon that has been implicated in the CSC hypothesis. It is defined as a remarkably long latency period between removal of the primary tumor and the successive distant relapse in a cancer patient who has been otherwise free of cancer

(Uhr and Pantel 2011). For example, in breast cancer, clinical observations suggest that a large proportion of cancer survivors have residual cancer cells for decades but can remain clinically cancer-free for their lifetime, because the remaining cells persist in a dormant state (Karrison et al. 1999). It has further been postulated that also here slow-cycling or even quiescent CSCs are the seeds for delayed metastatic outgrowth (Uhr and Pantel 2011). Therefore, CTCs that are released into the blood circulation might contain a minor subgroup of stem-like cells which get stuck in secondary organs and remain in a quiescent state for many years. Later, depending also on environmental factors, these founder cells or only a subpopulation of them can start to cycle again and to form micro- and macrometastases in the secondary tissue (Uhr and Pantel 2011). Interestingly, in preclinical models, it has been shown that during cancer progression, individual CTC that has reached secondary organs can occupy existing stem cell niches, a specialized microenvironment that regulate cell differentiation and cell-cycle entry of resident stem cells in the physiological context. Inside the stem cell niches, the disseminated tumor cells have to preserve and withstand a hostile environment to survive. Increasing evidence from functional studies suggests that these disseminated cells have to develop stem-like characteristics in order to persist in the stem cell niches (Lander et al. 2012). Once they have established themselves in the niches, they do not rely exclusively on cell-intrinsic events but instead depend heavily on the microenvironment to control the dormant state as well as maintain proliferative activity and cellular fitness. Consequently, it has been suggested that signaling pathways that sustain the CSC phenotype are attractive targets for innovative therapeutic strategies. Such substances that inhibit interaction of quiescent cells with the stem cell niches could make these cells vulnerable for chemotherapy (Lander et al. 2012). In summary, the CSC hypothesis yields important suggestions for cancer therapy and tumor diagnosis because it has implications for major clinical problems including therapy resistance and distant metastasis formation. Thus, just like for EMT traits, great attention has been paid on comprehensive analyses of CTC heterogeneity and identification of stem-like CTC subpopulations in patient-derived blood samples.

3 Principles of Enrichment and Analysis for Mesenchymal-like CTC

At present, various assays utilizing different methodical concepts are used for the enrichment and characterization of CTC. Many platforms are using antibodies that are recognizing epitopes of epithelial- or cancer-specific proteins. These antibodies are usually coupled to immunomagnetic beads or nanoparticles allowing CTC enrichment based on the expression of the corresponding antigens (Schilling et al. 2012; Alix-Panabieres and Pantel 2014; Hegemann et al. 2016). One of the mostly utilized antigens is the EpCAM protein. This protein is expressed by epithelial tumor cells but is absent on benign blood cells. However, it has been demonstrated by several groups that a subpopulation of CTC has diminished or deficient EpCAM

expression. It is largely considered that these cells have lost EpCAM expression while undergoing EMT. Such tumor cells with mesenchymal features and lacking EpCAM expression can evidently not be detected by EpCAM-based enrichment platforms (Alix-Panabieres and Pantel 2014; Hyun et al. 2016). On the other hand, it has been revealed that EpCAM-positive cells can also be existent in the circulation of patients with benign colon disease questioning the application of EpCAM as a tumor-specificity marker (Pantel et al. 2012). For that reason, strong research efforts have aimed to develop analytical assays that can enrich CTC independent of their EpCAM protein expression. These methods commonly use physical and biological characteristics of tumor cells for CTC enrichment. In general, an assay for CTC enrichment should yield a high recovery rate of CTC as well as a high purity. Non-optimal purity or recovery is an issue with all currently used CTC platform, either EpCAM dependent or independent ones. Also loss of cells and long blood processing times should be avoided. A high recovery rate is required to avoid false-negative results and the loss of cells with particular biological and prognostic relevance. Still, there is lack of evidence that shows which characteristics of CTC leading to formation of metastasis and disease progression. To assess the efficiency of any CTC platform, reliable data on recovery rates is always mandatory. The most commonly implemented approach for defining recovery rates of CTC is the spiking of definite numbers of cultured tumor cells into the peripheral blood of a healthy donors with subsequent quantification by the particular method (given that the approach allows quantification) (Todenhofer et al. 2017; Weissenstein et al. 2012). A common problem with using individual cells from stable cell lines is that they commonly do not entirely represent the diversity and heterogeneity of tumors (Park et al. 2014). Conversely, studies claiming an increased sensitivity of a platform due to higher detection rates compared to another platform observed in patients' samples have to be considered with caution, because the real number of CTC cannot certainly be determined (Andreopoulou et al. 2012; Van der Auwera et al. 2010). This is a common and general limitation of all CTC detection systems. The other key feature of a practical CTC assay is a high CTC purity which is necessary to reduce the requirement of downstream analyses for the identification of CTC within the enriched population. Such optional downstream methods certainly decrease the CTC recovery rate and may limit the opportunities for classification of biologic the traits of CTC, e.g., short- and long-term culture, in vitro drug testing, or single-cell transcriptome analysis (Alix-Panabieres et al. 2016). For instance, a microfluidic enrichment platform based on deformability and size of tumor cells provides a 100% recovery. But due to contaminating leukocytes in the outflow channel with a ratio of 10 leukocytes per CTC a purity of only 5–10% can be achieved. In this example, further analysis is obligatory in order to exactly identify CTC. One option for a subsequent complementary analysis could be immune-cytochemical staining of potential CTC with epithelial or tumor-specific markers. As such, most commonly used marker immuno-cytochemical CTC detection is protein that is exclusively expressed on cells of an epithelial origin, such as cytokeratins. Just like the EpCAM–protein, these markers can be diminished in cells that have underwent

EMT (Barriere et al. 2014). To date, information on non-epithelial CTC-specific marker is short. Moreover, the benefit of a marker-independent enrichment strategy compared to a marker-based enrichment goes to some extent lost when using a specific marker in a second validation step. One major drawback of immuno-cytochemical CTC analysis is that the required cell fixation ultimately killing the cells. Unfortunately for any in vitro analysis like drug testing or cultivation, which have a high relevance for improving the understanding of the biology of CTCs, cell viability is crucial (Alix-Panabieres et al. 2016). For that reason, a high purity after enrichment is critical to prevent the need for additional analyses. Nevertheless, antigen-dependent enrichment methods that allow a specific enrichment of CTC also with mesenchymal phenotype have not been widely employed so far. Typical markers of mesenchymal differentiation such as N-cadherin and vimentin are commonly expressed also on peripheral blood mononuclear cells (PBMCs). Consequently, these markers are not appropriate for specific CTC enrichment. An alternative concept allowing enrichment of malignant mesenchymal-like CTC is depletion of CD45-expressing leukocytes from the analyzed cell suspension. This negative selection is, in addition to the positive selection of EpCAM, also implemented in the CellSearch platform. However, current studies have demonstrated that methods exclusively based on depletion of CD45-positive cells still yield a high number of contaminates consisting of CD45-negative PBMC. This impurity of benign cells considerably affects the outcome of downstream applications aiming to specifically identify and characterize CTC. Thus far, there is no widely used and validated marker for specific enrichment of CTC with mesenchymal phenotype. Although different methods have been proposed, none of them has convincingly shown to allow a specific enrichment of cancer cells from blood samples. However, to detect tumor cells with mesenchymal characteristics, the choice of appropriate markers is of major importance. In general, different methodologies are possible. Foremost, it has been demonstrated that the principle of assessing epithelial and mesenchymal markers in parallel is a practicable approach. However, prerequisite is that the analyzed CTC has not switched completely to a mesenchymal differentiation and still shows at least residual expression of epithelial markers. The most commonly used epithelial-specific proteins in this context is cytokeratin (Kallergi et al. 2011) even though down-regulation of epithelial-specific cytokeratins is a feature frequently that occurs during EMT (Lamouille et al. 2014). For that reason, a simultaneous analysis of cytokeratins and mesenchymal markers is likely to detect only intermediate state of differentiation and not CTC that has switched completely to a mesenchymal phenotype. However, the sole detection of mesenchymal markers bears the risk to identify non-tumor cells expressing mesenchymal markers. Leukocytes have been shown to express mesenchymal markers such as vimentin (Wu et al. 2015). Thus, another possible methodology is to perform a negative depletion for leukocyte-specific proteins in combination with the detection of mesenchymal markers (Wu et al. 2015; Dinney et al. 2014).

4 Prognostic and Clinical Relevance of Mesenchymal Marker Expression on CTC

In the setting of identifying CTC with mesenchymal differentiation, several proteins have been discussed and used most frequently as markers for CTC analysis. In mesenchymal cells, the vimentin protein is a major component of the cytoskeleton and acts as a molecular determinant for cellular motility. As such, it regulates cellular integrity, stabilizes interactions within the cytoskeleton, and mediates durability toward mechanical stress. In various preclinical and translational studies, it has been shown that the expression of vimentin in tumor cells is associated with the development of metastases (Hu et al. 2004; Zelenko et al. 2017). The relevance of vimentin expression in CTC has also been addressed by various studies. Particularly in blood samples from advanced breast cancer patients, vimentin-positive CTCs were found more frequently compared to early cancer patients. In another study, also co-expression of vimentin with the EMT-promoting transcription factor TWIST has been described (Kallergi et al. 2011). Vimentin-positive CTC has been also described as poor prognostic factor in different cancers including prostate cancer and pancreatic cancer (Lindsay et al. 2016; Poruk et al. 2016). However, one of the main restrictions for the usage of vimentin as a marker protein is that intracellular expression of vimentin is not only present in tumor cells but also in benign blood cells (Gorges et al. 2016). From there, the identification of vimentin at the cell membrane, which is typical for tumor cells, is of particular importance (Mitra et al. 2015; Satelli et al. 2014). For instance, CTC derived from sarcoma patients can be enriched on the basis of cell-surface expression of vimentin. Also in other solid tumor types, the expression of cell-surface vimentin has been shown to correlate with therapy response and outcome (Satelli et al. 2015a, b). Like vimentin also N-cadherin is an important structural component of mesenchymal cells, acting at adherens junction protein complexes at cell–cell connections. It is a generally accepted marker of mesenchymal differentiation. Similar to other mesenchymal markers, elevated N-cadherin expression correlates with increased metastatic potential of tumors and poor patient prognosis (Hui et al. 2013; Nieman et al. 1999; Yi et al. 2014). On the other hand, the knowledge on the role of N-cadherin expression in circulating tumor cells is ill-defined. Though in a pilot study, Armstrong et al. could show in a cohort of 10 patients with breast cancer and 10 patients with metastatic castration-resistant prostate cancer (CRPC) that after enrichment for EpCAM-positive cells, 84% (CRPC) and 82% of CTC express N-cadherin (Armstrong et al. 2011). However, in most patients, N-cadherin-positive CTC showed also an abundant E-cadherin protein expression, indicating that these cells own an intermediate state of cellular differentiation. In a study evaluating the expression of N-cadherin together with expression of the stem-cell marker CD133 in CTCs of 26 patients with metastatic breast cancer, N-cadherin expression was present in less than a third of CTC, emphasizing the dependence of the expression of these markers not only on the patient cohorts but also the methodology used for analysis and CTC enrichment (Bock et al. 2014). ZEB1 is a zinc finger and homeodomain

transcription factor which biological function is highly relevant for cell differentiation and which is also related to tumor cell dissemination and metastatic progression (Bourcy et al. 2016). Recent preclinical models strongly suggest that inactivation of ZEB1 function reduces the metastatic potential of tumor cells (Bourcy et al. 2016; Zhang et al. 2015). Lately, ZEB1 expression has been used to identify a subpopulation of CTC with a mesenchymal phenotype and has been found to be co-expression of other EMT-related genes (Gorges et al. 2016). In a study evaluating KRAS mutations in CTC from pancreatic cancer patients, after depletion of CD45-positive white blood cells, ZEB1-expressing non-blood cells were defined as CTC (Kulemann et al. 2016). Interestingly, the majority of these ZEB1-expressing CTC also expressed CK19 as an epithelial marker, and only the minority of cancer patients showed only ZEB1 expression on CTC. Although limited by a small number of cases, ZEB1 expression was correlated with worse overall patient survival. Cytokeratin-negative, ZEB1-positive cells had a poorer outcome than double-positive cells. By transcript analysis of CD45-depleted PBMC, no CTC with ZEB1 expression was identified in a cohort of 102 patients with early breast cancer, whereas other EMT-associated transcripts, such as SLUG, were expressed, whereas applying transcript analysis of CD45-depleted PBMC from healthy donors, the authors could show that in benign cells a background expression of ZEB1 was also present. In accordance with this, another study in breast cancer showed that ZEB1 expression levels in EPCAM/CD326-positive CTC do not have higher levels of ZEB1 than CD45-positive white blood cells (Giordano et al. 2012). This has to be taken into consideration when interpreting the expression of ZEB1 in PBMC. TWIST1 is another transcription factor belonging to the basic helix–loop–helix family. Just like ZEB1, the TWIST1 protein is involved in cell lineage determination and differentiation. Mutations of the *TWIST1* gene have been linked to development of the Sezary syndrome (Howard et al. 1997). In the context of cancer progression, the TWIST1 has been discovered as important contributor to the process of EMT and the development of metastases (Zhu et al. 2016). TWIST1 is a transcriptional repressor of the *CDH1* gene coding for E-cadherin, which is a widely used marker for an epithelial phenotype (Vesuna et al. 2008). High expression of *TWIST1* correlates with poor survival in various cancer types (Riaz et al. 2012; Wushou et al. 2014). On the other hand, inhibition of TWIST protein function leads to reduced tumor growth and metastatic progression in preclinical models (Finlay et al. 2015; Khan et al. 2015). TWIST1 is also key factor of the Adnatest® EMT kit, which has been one of the first commercially available assays for detection of cells with a mesenchymal differentiation. After cell enrichment of EPCAM, EGFR or HER2-positive cells with immunomagnetic beads, a transcript analysis is performed for *TWIST1*, *AKT2,* and *PIK3CA*. Using this technology to detect CTC in blood samples from breast cancer patients, it was shown that particularly in advanced breast cancer, a significant proportion of patients have CTC with mesenchymal characteristics, which was indicated by gene expression of *TWIST1*, *AKT2* and *PIK3CA* and that neoadjuvant chemotherapy might be ineffective in eliminating these CTCs (Aktas et al. 2009; Mego et al. 2012). Also in bladder cancer patients, it was shown that 12.5% of patients

suffering metastatic progression have CTC positive for TWIST1 expression in comparison to non-metastatic bladder cancer patients (Todenhofer et al. 2016). Papadaki et al. examined TWIST1 protein expression in CTC by immunocytochemistry in blood samples of patients with early and metastatic breast cancer and observed that also the subcellular localization of the TWIST1 protein have clinical implications. In their study, nuclear TWIST1 localization in CTC was increased in the metastatic setting, whereas cytoplasmic TWIST1 localization was more frequent in CTC from early breast cancer (Papadaki et al. 2014). In general, similar to vimentin, TWIST1 has been also described to be expressed by white blood cells. Therefore, we recommend that assessment of TWIST1 expression should be combined with assessment of other markers or negative depletion of CD45-positive cells to minimize false-positive results (Li et al. 2010; Merindol et al. 2014). The PI3K/Akt/mTOR pathway is often constitutively activated and thus an attractive therapeutic target in a variety of malignancies including renal cell carcinoma. Being activated by PI3K, the Akt kinase has a wide range of downstream effects propelling tumor cell growth, invasion, and metastatic progression (Merindol et al. 2014). One important consequence of active Akt in the process of EMT is the suppression of E-cadherin (Barber et al. 2015; Larue and Bellacosa 2005). Transcript analysis of *AKT2* and *PIK3CA* are two key components of the above-mentioned Adnatest® assay for detection of CTCs associated with epithelial–mesenchymal transition and cancer cell stemness (Todenhofer et al. 2016). Likewise immuno-cytochemical analysis of Akt phosphorylation or PI3K phosphorylation showed positive CTC in >80% in blood samples from breast cancer patients with early and late stage breast cancer. Schneck et al. assessed activating mutations in the *PIK3CA* gene in CTC from breast cancer patients. Here, 7 out of 44 patients showed a SNP within the *PIK3CA* gene (Schneck et al. 2013). Also in metastatic colorectal cancer patients, Ning et al. observed a significantly inferior survival of patients with CTC-expressing PI3K-alpha or AKT2 (Ning et al. 2016).

5 Principles of Enrichment and Analysis for Stem-like CTC

Motivated by the specious benefits to specifically target subgroups of tumor cell populations with stem-like traits, different studies aimed to functionally characterize CSCs and also to prove the CSC hypothesis in cancer patients. Fundamental studies in hematopoietic malignancies recognized populations of CSC that could be serially transplanted into NOD-SCID mice and resulted in leukemia, whereas this phenotype was not seen in more differentiated leukemic cells. These CSC subsets can be prospectively detected and enriched by expression of characteristic cell-surface proteins. The common approach for the isolation of CSC is flow-cytometric fractionation of tumor cells using stem-cell-specific cell-surface markers, followed by their implantation into NOD-SCID mice to assess their tumorigenic potential

(Shipitsin and Polyak 2008). Utilizing this methodical approach, different research groups have succeeded in establishing subgroups of tumor cells with stem-cell-like characteristics and tumorigenic potential from different solid tumor entities. Nonetheless, in solid tumor entities, the differentiation hierarchy and expression pattern of putative stem cell markers are to a lesser extent characterized than in the hematopoietic malignancies (Shipitsin and Polyak 2008). In spite of incomplete understanding of stem-like traits of epithelial tumor cells, the most commonly used markers to identify CSCs from solid tumors are the proteins CD24, CD44, CD133, and ALDH1. At first in 2003, using xenograft transplantation model, Al-Hajj and colleagues were able to show that only a small subset of breast cancer cells had the ability to form new tumors in immune-deficient mice. In their study, they used flow-cytometric enrichment of CD44-high/CD24-low tumor cells to prospectively identify and isolate potential CSCs. Based on this principle, they were able to discriminate between tumor-initiating and non-tumorigenic cancer cells. These findings are in line with the postulated phenotype that only tumor cells with stem-like features are driving carcinogenesis. Moreover, the CD44-high/CD24-low expression pattern of mammary tumor cells can be used to enrich stem cells with multipotent differentiation ability (Al-Hajj et al. 2003). Patrawala and colleagues published a comparable xenograft transplantation model of prostate cancer cells. However, these authors used a simplified enriched strategy, which was solely based on CD44 expression. Nevertheless, they were able to show that CD44-high prostate cancer cell population is more proliferative, clonogenic, tumorigenic, and metastatic than the subgroup of CD44-low cells. Succeeding functional studies analyzing the properties of the CD44-high prostate cancer cells showed that these cells indeed possess certain intrinsic properties of metastatic progenitor cells (Patrawala et al. 2006). While not entirely applicable for breast- and prostate-derived CSC enrichment, expression of CD133 can also be applied to isolate stem-like tumor cells from different cancerous tissues including glioblastoma and colorectal tumors (Galli et al. 2004; O'Brien et al. 2007). Ginestier and colleagues demonstrated that also expression and activity of ALDH1 enable the detection and enrichment of both normal and tumor mammary stem cells in vitro, in vivo, and in situ in fixed tissues (Ginestier et al. 2007). Yet, all described studies enriched the putative CSC population from primary tumor tissue or pleura effusions but not from patient-derived blood samples with might contain CTC. In contrast, Baccelli et al. aimed in a pioneering study to specifically isolate MICs from blood samples of breast cancer patients to test the hypothesis whether CTC really contains subpopulation of highly aggressive CSCs that are capable to from distant metastasis at secondary organs. The authors took blood samples from luminal breast cancer patients with known CTC count. These blood samples were depleted for hematopoietic cells, and potential CSCs were further enriched for expression of surface markers EPCAM, CD44, CD47, and MET. Afterward, the enriched cell populations were used in a xenograft assay for implantation into the bone marrow cavity of sublethally irradiated mice. In this experiment, the putative CSC subpopulation formed distant bone, lung, and liver metastases in mice confirming that primary human luminal breast cancer CTC really contains MICs. This result was also validated in a small

cohort of patients with metastases, here the number of CTC, with positive EPCAM, CD44, CD47, and MET but not with merely EPCAM expression correlated with poor overall survival and increased number of metastatic sites. These results defined for the first time that functional circulating MICs exist among total CTC in blood samples from cancer patients (Baccelli et al. 2013). Lu et al. published another study aiming to directly identify and enrich invasive CTC from blood samples of breast cancer patients. To isolate CTC with an invasive phenotype from patient-derived blood samples, the authors used a functional cell separation method based on collagen adhesion. By using this approach, the authors were able to isolate viable CTC from blood of stage one to stage three breast cancer patients. Subsequent gene expression and flow-cytometric analyses on the captured and invasive CTC showed the presence of distinct cellular subgroups including an epithelial lineage and stem or progenitor cells (Lu et al. 2010). More recently, also a stable CTC cell lineout of the blood of a colorectal cancer patient designated CTC-MCC-41 has successfully been established. Systematic analysis of this cell line revealed that it resembles properties of the original primary and displays a stable phenotype featuring an intermediate epithelial/mesenchymal phenotype and stem-cell-like characteristics. Functional in vitro analysis further revealed that CTC-MCC-41 cells induced rapidly endothelial cell tube formation as well as tumor formation in a xenograft implantation assay in immune-deficient mice. Collectively, the establishment of this colon cancer-derived CTC line enables various functional analyses on the CTC phenotype and applications for in vitro and in vivo also drug testing (Cayrefourcq et al. 2015). Still, there is also evidence that the CSC model cannot be applied to explain the biology of all tumors. For example, a proof-of-concept study for ex vivo culture and characterization of CTC from patient, which was conducted to noninvasively supervise the shifting patterns of drug susceptibility in breast cancer patients, did not observe elevated expression of defined stem-cell-related signatures in CTC cultures, although most of the analyzed CTC cell lines were tumorigenic in xenograft assays and derived from metastatic patients (Yu et al. 2014).

6 Prognostic and Clinical Relevance of Stem Cell Marker Expression on CTC

The CSC hypothesis endorses important consequences for the detection and clinical relevance of CTC. Firstly, if a subgroup of biologically unique CSCs really exists, then tumor cells shed from the primary lesion lacking the stem cell properties will not be competent to initiate self-propagating metastases, regardless of their differentiation status or proliferative potential. Furthermore, curative therapy will need complete elimination of the entire CSC population. Patients who show an initial response to treatment may ultimately relapse if even a small number of CSCs persists (Marsden et al. 2009). Thus, the detection of CTC in peripheral blood samples with stem-like characteristics may represent a valuable diagnostic approach

to direct treatment decisions. In addition, also functional analysis of stem-like CTC could expand the knowledge about mechanisms of tumorigenesis, dormancy, and metastatic outgrowth. From there signaling pathways that sustain the CSC phenotype represent attractive targets for the establishment of innovative treatment strategies (Lander et al. 2012). Several studies aimed to determine and further characterize a stem-like subpopulation of CTC. Following some of these studies from different tumor entities are briefly described. In 2009, the first study that aimed to analyze stem cell characteristics of CTC in blood samples from breast cancer patients was published by Aktas and colleagues. The authors assessed 226 blood samples from 39 metastatic breast cancer patients during a follow-up therapy for the expression of the stem cell marker ALDH1 beside other EMT markers and correlated results with CTC count and therapy response. In this study, in blood samples from CTC-negative patients, ALDH1 mRNA was detected in 14% of analyzed cases, whereas in the CTC-positive group, ALDH1 was found in 69% of blood samples. In non-responders, ALDH1 transcripts were found in 44% of patients; in responders, the rate was in contrast only 5%. This data implies that a large proportion of CTC of metastatic breast cancer patients indeed shows tumor stem cell features (Aktas et al. 2009). Further studies also estimated expression and activity of ALDH1 in CTC in blood samples from breast cancer patients. Papadaki et al. found by applying triple immunofluorescence staining of individual CTC with anti-cytokeratin, anti-ALDH1, and anti-TWIST antibodies that CTC from patients with metastatic breast cancer frequently expresses high amounts of ALDH1 protein. They could further show a nuclear localization of the EMT-related transcription factor TWIST. This indicates that these CTCs exhibit stem-like characteristics and may prevail during cancer progression (Papadaki et al. 2014). In addition, in a prospective study published by Giordano et al., a comprehensive methodology was applied to evaluate putative CSC characteristics of CTC in 28 patients with HER2-positive metastatic breast cancer. The authors enriched CTCs from peripheral blood using CD326 and CD45 depletion. These cells were analyzed using multiparameter flow cytometry for ALDH activity and for expression of stem cell markers CD24, CD44, and CD133. Additionally, also transcript analysis of EMT marker expression was done in the purified cells. Here, the enriched CTC fraction from patients with elevated expression of EMT-related transcripts had also significantly higher percentage of ALDH and CD133-positive cells in their blood than did patients with normal expression of EMT markers, suggesting that patients with HER2-positive metastatic breast cancer bear CTCs with EMT and CSC characteristics (Giordano et al. 2012). Besides surface expression of CD44 and CD24 proteins has been successfully utilized to enrich CTC subpopulations with CSC features from blood samples of breast cancer patients. To specifically isolate CTC subgroups that are associated with tumor dormancy, Vishnoi and colleagues enriched for lacking EpCAM and CD24 expression but positivity for CD44 expression from peripheral blood of patients diagnosed with or without breast cancer brain metastasis. They further combined their analysis with assessment of uPAR and Integrin-β1 expression. These two markers are implicated to be directly involved in control of breast cancer dormancy. CTCs isolated by this method were successfully

cultured as three-dimensional tumorspheres. Interestingly, tumor cell growth and invasiveness of these CTC cultures were distinctive upon combinatorial expression of uPAR and Integrin-β1. Thus, this methodology may improve abilities to prospectively recognize patients who may be at high risk of developing breast cancer brain metastasis (Vishnoi et al. 2015). Studies on stem cells in colonic crypts have added substantial knowledge to the understanding of stem cell biology in epithelial tissues. The Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) is strongly expressed in multipotent stem cells compared to their immediate progeny, and thus, it is a well-established marker for stem cells in the small intestine and colon (Barker et al. 2007). The clinical relevance of LGR5 mRNA expression as biomarker in peripheral blood of colorectal cancer patients was evaluated by Valladares-Ayerbes in 54 patients and 19 controls. They found that *LGR5* gene expression was significantly higher in blood samples from colorectal cancer patients compared to healthy controls. Furthermore, raised LGR5 mRNA expression in the evaluated blood samples correlated with metastasis, high-grade, and poor overall patient survival. These results point out that the analysis of *LGR5* mRNA in peripheral blood might reflect the presence of stem-cell-like CTCs in blood samples taken from colorectal cancer patients (Valladares-Ayerbes et al. 2012). CD133 is another well-established stem cell marker for colorectal CSCs and has been used to enrich and describe potential CSC subsets in blood samples from colorectal cancer patients. Out of blood samples from seven colorectal cancer patients, Malara and colleagues were able to separate heterogeneous CTC populations. They distinguished two distinct subgroups of CTCs according to CD133 expression, which were also associated with different clinical outcome. Thus, patients with prevalence of putative circulating cancer stem cells showing CD133 expression have a lower overall survival (Malara et al. 2016). Besides, Pilati et al. retrospectively evaluated prospectively collected preoperative blood samples to establish putative circulating biomarkers in patients undergoing complete resection of metastatic colorectal cancer to the liver. Among seven analyzed genes, the expression of CD133 was found to be the only independent predictor of patient survival. The authors concluded that CD133-positive CTCs may represent a suitable prognostic marker to stratify the risk of patients who undergo liver resection for CRC metastasis, which opens the avenue to identifying and potentially monitoring the patients who are most likely to benefit from adjuvant treatments (Pilati et al. 2012). Other studies found comparable results by analyzing blood samples of prostate cancer patients. For instance, Armstrong et al. assessed expression of the stem-cell marker CD133 together with expression of EMT markers by immunocytochemistry in CTCs from 41 patients with castration-resistant prostate cancer. They found that most analyzed CTCs co-express the stem cell marker CD133 together with epithelial proteins such as EpCAM, cytokeratins, and E-cadherin and also with mesenchymal proteins including vimentin, N-cadherin, and O-cadherin. Based on these findings, the authors suggested that stem-like CTCs may be enriched among patients with metastatic epithelial tumors which might account for therapy resistance often seen in this patient group (Armstrong et al. 2011). To test whether detection of EMT and stem-cell-associated mRNA expression in peripheral

blood derived from metastatic prostate cancer patients could complement plain CTC enumeration, Chang and colleagues set up a quantitative PCR-based assay. In total, they analyzed peripheral blood from 70 patients and enumerated CTC in these blood samples using the CellSearch system. In parallel gene expression of prostate stem-cell-related genes, *ABCG2, CD133,* and *PSCA,* and EMT-related genes *TWIST1* and *VIM* were analyzed by quantitative PCR. In this study, it was found that stem-like cell gene expression indicated poor prognosis, whereas EMT-related expression did not. Also for 40 patients with favorable CTC enumeration, positive stem cell gene expression suggested a poor prognosis. As a result, detection of peripheral blood stem cell gene expression could complement CTC enumeration in predicting overall survival and treatment effects in metastatic prostate cancer patients (Chang et al. 2015). Also in blood samples from endometrial cancer patients, CTC populations with co-expression of EMT and stem cell markers have been detected. In high-risk endometrial cancer patients, CTC that was enriched based on EpCAM expression exhibited a plasticity phenotype marked by the expression of the EMT markers and expression of stem cell markers ALDH and CD44 (Alonso-Alconada et al. 2014). EpCAM-enriched CTCs with stem-cell-like phenotypes were identified in blood samples taken from hepatocellular carcinoma patients, too. These CTCs showed co-expression of EMT markers with the cancer stem cell markers CD133 and ABCG2. In addition, this expression pattern was also accompanied by activation of the Wnt pathway, high tumorigenic potential, and low apoptotic propensity. From there, this CTC subpopulation may constitute the tumor-initiating subpopulation in hepatocellular carcinoma specimens, and its detection may serve as a real-time parameter for monitoring treatment response (Sun et al. 2013). Schulze et al. started EpCAM-based isolation of stem-cell-like CTC from hepatocellular carcinoma patients. Corresponding to the study of Sun and colleagues, they reported the detection of EpCAM-positive, stem-like CTCs in patients with intermediate or advanced hepatocellular carcinoma. They furthermore showed that the detection of these CTCs had prognostic value for overall survival with possible implications for future treatment stratification (Schulze et al. 2013). Also in blood samples taken from non-small cell lung cancer patients, ALDH1 expression was detected, indicating the presence of stem-like CTCs in this patient group (Hanssen et al. 2016). Taken together, several studies encompassing tumor patients of different solid tumor entities have investigated stem cell marker expression in CTCs and directly in peripheral blood samples, respectively. In most studies, the detection of increased expression of CSC markers like CD133, CD44, and ALDH1 was correlated with poor patient outcome. This suggests that among the entire CTC population, a rare subpopulation of stem-like CTCs really exists. Still, there is evidence that progression of some tumors cannot convincingly be explained by the stem cell model. For instance, in one study done by Quintana et al., the authors have not detected a particular subpopulation of melanoma cells without tumorigenic potential. None of 22 heterogeneously expressed markers including enriched tumorigenic cells. Some melanomas metastasized in mice, irrespective of putative stem cell marker expression. Also many markers appeared to be reversibly expressed by tumorigenic melanoma cells (Quintana et al. 2010).

Additionally, the real impact of tumor-initiating cells that show stem cell marker expression is still poorly defined. For instance, in human metastatic colon cancer it was shown that CD133 expression alone is not sufficient to identify the complete subpopulation of epithelial and tumor-initiating cells. In a study done by Shmelkov et al., CD133-positive as well as CD133-negative metastatic tumor subpopulations formed colonospheres in in vitro cultures and were capable of long-term tumorigenesis in a serial xenotransplantation model. This indicates that CD133 expression is not limited to intestinal stem or cancer-initiating cells (Shmelkov et al. 2008). Moreover, latest studies based on genetic lineage tracing describe various strategies employed by normal epithelial stem cell hierarchies to replace damaged or lost stem cells (Greulich and Simons 2016). These findings challenge the anticipated biological relevance of CSC because the CSC model of tumor cell hierarchies proposes that commitment and differentiation occur unidirectional. Furthermore, plasticity within a tumor cell population might be more common than presupposed in the classical CSC model. Irrespective of the underlying biological principles is the detection of aggressive CTC subsets in peripheral blood from cancer patients of high clinical interest. Directly evaluating the effect of systemic cancer therapy by sequential assessment of potential subpopulation as drivers of metastatic progression is feasible, but a proper methodology requires better specificity and sensitivity as mere CTC enumeration. Precise CSC identification is certainly needed to implement the CSC concept into clinical practice, and validated protocols for liquid biopsies could pave the way for interventional clinical studies on treatment stratification in the future.

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Circulating Tumor Cell Enrichment Technologies

Mert Boya, Chia-Heng Chu, Ruxiu Liu, Tevhide Ozkaya-Ahmadov and Ali Fatih Sarioglu

1 Introduction

Cancer metastasizes through tumor cells transported from the primary tumor to distant organs via lymphatic and hematogenous circulation. These migratory cancer cells, called circulating tumor cells (CTCs), detach from the primary tumor, intravasate through leaky vessels, remain viable in the circulation and extravasate at a distant site, where they proliferate to form a new tumor (Fidler 2003). As such, the isolation of CTCs from blood samples and their subsequent analysis are of paramount importance not only for understanding cancer metastasis at the cellular level but also for clinical management of the disease. For example, in vitro propagation of enriched CTCs (Yu et al. 2014) will potentially be instrumental in functional studies on cancer metastasis in animal models, studying pharmacodynamics of the tumor reaction to various treatments and in developing therapies specifically targeting the metastasis process. Clinical utility of CTC detection has already been demonstrated in cancer diagnosis and prognosis (Cristofanilli et al. 2004; van de Stolpe et al. 2011), identifying stages of the disease (Budd et al. 2006), and monitoring patient response to the therapy (Al-Mehdi et al. 2000; De Bono et al. 2008; Hayes et al. 2006). Moreover, the molecular analysis of CTCs (Jahr et al. 2001; Shaffer et al. 2007; Smirnov et al. 2005) isolated from cancer patient blood samples holds great promise in guiding newly developed targeted therapies without invasive biopsies.

Detection of CTCs from patient samples is a technological challenge, because CTCs are mixed with host cells in circulation and CTC detection technologies need to differentiate tumor cells from the background and enrich them in a form compatible with downstream assays. Targeting of tumor cells is difficult because

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biomarkers used to identify CTCs are not specific and hence cannot fully exclude blood cells (Sawyers 2008). Also, due to the heterogeneity of tumor cells, even within the same patient, there does not exist a single biomarker that can universally be used to identify all CTCs (Powell et al. 2012). Such challenges make the specificity an important design parameter and performance metric for CTC enrichment technologies in minimizing false-positive/negative results. Another challenge for CTC enrichment is the extreme scarcity of CTCs in blood as there are approximately 1–10 CTCs (as compared to billions of normal blood cells) for each milliliter of blood (Alix-Panabières and Pantel 2014; Pantel and Alix-Panabières 2010). Therefore, for reliable CTC enrichment, it is often not an option to miss even a single CTC. This is especially true if CTC enrichment technologies were to be used for detection of early stage tumors that produce even fewer CTCs (Lucci et al. 2012). Therefore, the sensitivity is another crucial design and performance parameter for CTC enrichment technologies.

The immense potential of CTCs combined with technological challenges in their detection created an active research area focusing on the development of tools to detect CTCs in cancer patient blood samples (Nagrath et al. 2007). As early as 1950s, scientists attempted to enrich CTCs by utilizing the density gradient centrifugation, where cells are separated under centrifugal forces. Based on the contrast in sedimentation coefficient, which is directly proportional to cell size and density as defined by Stoke's law of sedimentation (Katkov and Mazur 1999), the density gradient centrifugation makes cells with similar properties form layers in a laboratory tube (Lu et al. 2015). This batch enrichment process was first demonstrated by Fawcett et al. (Fawcett et al. 1950) using albumin as the medium to separate erythrocytes, leukocytes, and malignant tumor cells into different layers. However, due to the cost and complexity of albumin preparation, this method was not widely used. Later, Seal (1959) introduced the silicon blending oil medium and successfully observed CTCs in 53% (25 out of 47) of the gastrointestinal tract cancer patients screened. Although the sensitivity was not sufficient for clinical applications, these studies demonstrated the potential of centrifugation-based techniques in CTC enrichment and were later followed by Percoll and Ficoll-Paque density centrifugation techniques (Li et al. 2017; Yoo et al. 2016; Krishnamurthy et al. 2013).

Micro- and nanofabrication techniques, originally developed for integrated circuits, were later applied to biomedicine and led to important advances in CTC enrichment by enabling the development of microfluidic systems. Especially the introduction of soft lithography (Fujii 2002) allowed biomedical researchers, who are non-specialists in device fabrication, to quickly prototype, test, and optimize microfluidic systems, thereby significantly contributing to the innovation in the field. Since the seminal work by Nagrath et al. (2007) a decade ago, numerous microfluidic CTC isolation devices employing different enrichment principles (Fig. 1) have been developed (Ferreira et al. 2016). Microfluidic systems for CTC enrichment possess several advantages over traditional batch processes. First, microfluidic systems can be engineered to achieve deterministic screening of each and every cell in a sample within a controlled microenvironment, leading to lower

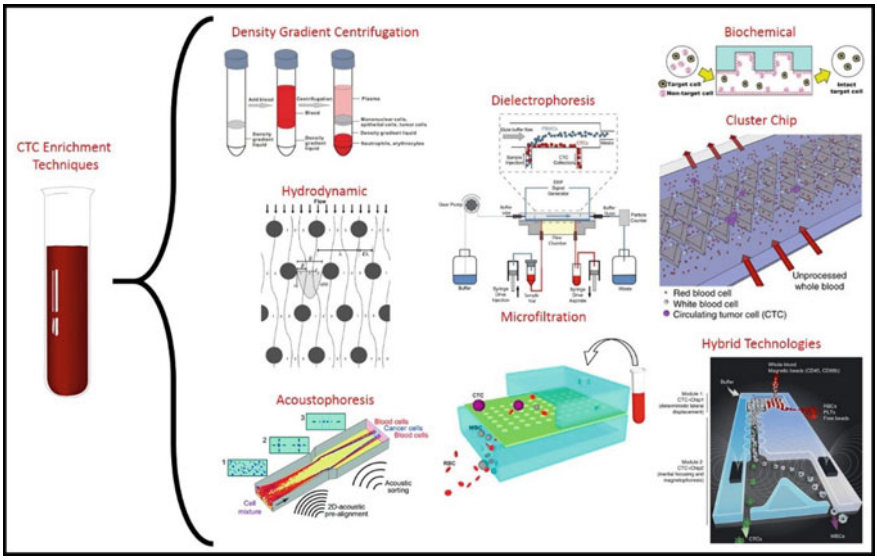

Fig. 1 Schematic showing different discrimination mechanisms employed in CTC enrichment. Figure panels adapted and reproduced from Antfolk et al. (2015) with permission from Creative Commons; Fan et al. (2015) with permission from Elsevier; Gupta et al. (2012) with permission from AIP Publishing; Hyun et al. (2013) with permission from American Chemical Society; Inglis et al. (2006) with permission from The Royal Society of Chemistry; Karabacak et al. (2014) with permission from Nature Publishing Group; Lu et al. (2015) with permission from Wiley; Sarioglu et al. (2015) with permission from Nature Publishing Group

cell loss and artifacts, and higher sensitivity. Second, microfluidic systems can utilize a variety of physical or chemical interaction forces as well as near field effects to discriminate tumor cells from others and hence can enable the discovery and application of non-traditional biomarkers for CTC enrichment. On the other hand, microfluidic devices are inherently slower than batch processing methods and therefore processing clinically relevant volumes of blood samples using these tools is a challenge.

As will be seen in this chapter, a technology can enrich CTCs either by targeting and directly isolating tumor cells (i.e., positive enrichment) or by targeting and selectively eliminating contaminating normal blood cells to leave the tumor cells behind (i.e., negative enrichment). The positive enrichment of cells relies on tumor-specific biomarkers and therefore leads to biased selection of a subset of CTCs given the heterogeneity among tumor cells (Lustberg et al. 2012). Negative enrichment-based technologies produce unbiased results by using established biomarkers for blood cells but suffer from low purity due to contaminating white blood cells that evaded depletion (Hyun et al. 2013; Ozkumur et al. 2013). This is problematic since enriched product purity is especially important to minimize the background noise in molecular assays (Lara et al. 2004; Yang et al. 2009).

Another important parameter that varies among different CTC enrichment technologies is the type and the extent of sample manipulation required for the enrichment process. Sample manipulation can include the pre-labeling of target tumor or blood cells (Karabacak et al. 2014), fixing of cells (Coumans et al. 2013), red blood cell lysis (Warkiani et al. 2016), pre-filtering (Tan et al. 2009), and sample dilution (Loutherback et al. 2012). While these processes often aim to aid the enrichment process by amplifying the contrast between the target and non-target cells, they may interfere with downstream assays, risk artifacts, and cell loss, and hence limit the practical applicability of the technology for certain applications. To ensure against such issues, CTC enrichment technologies that can process unmanipulated blood samples have also been developed (Lee et al. 2013b).

In this chapter, different CTC enrichment technologies will be divided into subcategories based on the enrichment principle and discussed in detail. First, we will discuss the technologies that make use of biophysical differences between CTCs and blood cells. As part of the biophysical-property-based isolation, we will specifically focus on microfiltration, hydrodynamic, acoustophoresis, and dielectrophoresis-based techniques. We will conclude this section by covering recently developed devices that specifically target CTC-clusters. Next, we will present CTC enrichment techniques based on biochemical property differences. We will specifically discuss the immunoaffinity-based negative and positive enrichment of CTCs using antibodies and aptamers. Finally, we will conclude by providing examples of hybrid tools that combine different enrichment mechanisms in a single system.

It should finally be noted that this chapter cannot possibly include all of innovative work in the field of CTC enrichment. Therefore, the references provided here are intended to be representative examples and should be interpreted by the reader as a starting point for further research in CTC enrichment technologies.

2 Biophysical Contrast-Based Enrichment of CTCs

The use of biophysical properties of cells has drawn great interest because of its reliance on the intrinsic contrast in cell properties and paved the way for the development of label-free devices to be used in CTC enrichment. Biophysical contrast in properties such as size, deformability, density, and electrical properties has become useful markers for distinguishing CTCs from other components of blood. This section will focus on the technologies exploiting the differences between biophysical properties of CTCs and blood cells for isolation and enrichment.

2.1 Microfiltration-Based Enrichment Techniques

Microfiltration-based enrichment techniques for tumor cell enrichment date back to mid-1960s (Seal 1964). The larger phenotype of tumor cells provided the ability for filtration-based enrichment methods to be used for CTC isolation (Fan et al. 2015). With the advancement of microfabrication techniques, sophisticated microfiltration systems have been developed. Commercially available ISET® (Isolation by Size of Epithelial Tumor cells) (Rarecell, Paris, France) utilizes track-etched polycarbonate membranes for size-based enrichment of tumor cells (Vona et al. 2000). The design includes 12 wells with 8-μm-diameter cylindrical pores for direct filtration of 10 mL of 1:10 diluted sample. Although it enriches tumor cells without damaging their morphology (Chinen et al. 2013), low specificity hinders its practicality. Similar to ISET®, ScreenCell® filtration device (ScreenCell, Paris, France) uses circular track-etched membranes with 6.5- or 7.5-μm pores for isolation of live or fixed cells, respectively (Desitter et al. 2011). The single-use and low-cost device comprises three different types: ScreenCell® Cyto, isolating fixed cells for cytological studies; ScreenCell® CC and ScreenCell® MB, isolating live cells for cell culture and molecular biology, respectively. Compared to ISET®, ScreenCell® has the advantage of enriching unfixed live cells; however, it also suffers from low specificity because of leukocyte contamination. The assessment of ScreenCell® Cyto was performed with blood samples collected from 76 patients with known or suspected lung cancer, and the technology has been proven to be a potential diagnostic blood test for lung cancer (Freidin et al. 2014).

Apart from track-etched membranes, other microfabricated filters are also used in filtration-based CTC enrichment. Photolithographically patterned microfilter CellSieve™ (Creatv MicroTech) contains 160,000 pores of 7 μm diameter, uniformly distributed over a 9-mm-diameter filtration area (Fig. 2a) (Adams et al. 2014). Tests were conducted using MCF-7 breast cancer cells spiked into 7.5 mL whole blood, and Adams et al. revealed that compared to track-etched counterparts, this design had, on average, 25 and 35% higher capture efficiencies for fixed and unfixed cells, respectively. Moreover, filter contamination was reduced more than tenfold from the mean value of 47,840 to 3920 white blood cells.

Despite the availability of commercialized filters, development of optimized microfilters for CTC enrichment is an active area of research. Zheng et al. employed reactive-ion etching (RIE) for etching parylene membrane to precisely control the size, geometry, and density of pores across the filter (Zheng et al. 2007). 1-cm-by-1-cm-square parylene sheet is etched to obtain 10-μm-diameter circular pores with a periodicity of 20 μm. According to the reported recovery rates, this parylene-based design outperformed the CellSearch® system, which is the only Food and Drug Administration (FDA) approved system used for monitoring patients with metastatic breast, prostate, and colorectal cancer (Lin et al. 2010). The parylene microfilter device identified CTCs from 51 of 57 patients with a >90% recovery rate. Despite its promising recovery rate, the autofluorescence of parylene material complicates its use for clinical applications (Lu et al. 2010a). Furthermore, in order to prevent cell lysis under shear forces that occur during filtration,

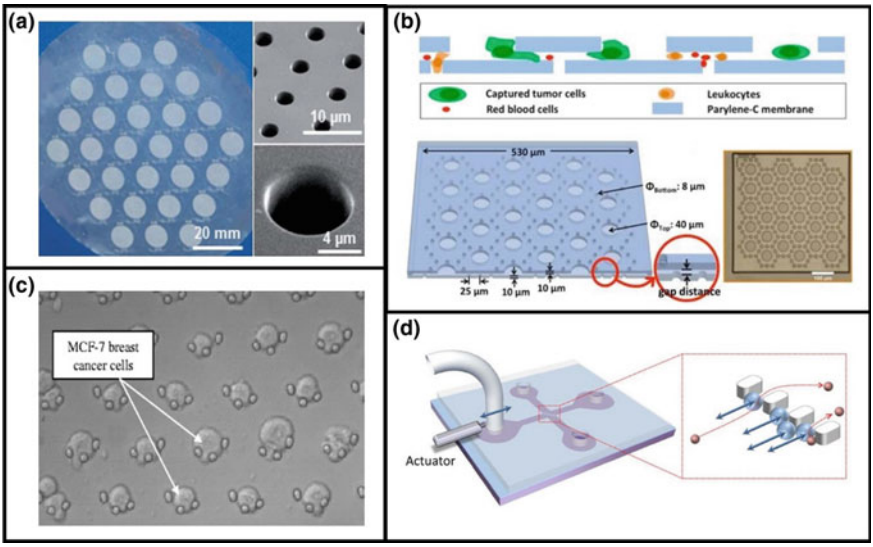

Fig. 2 **a** Image of a CellSieve device and scanning electron microscope (SEM) images of photolithographically defined pores. **b** 3D parylene separable bilayer microfilter and its cross-sectional view illustrating the working principle. **c** Crescent shaped isolation traps and captured MCF-7 cells by them. **d** Clog-free microfluidic chip and the magnified view of filtration area. Figure panels adapted and reproduced from Adams et al. (2014) with permission from The Royal Society of Chemistry; Tan et al. (2009) with permission from Springer; Yoon et al. (2016) with permission from Creative Commons; Zhou et al. (2014) with permission from Nature Publishing Group

pre-fixation of the blood sample is required, rendering it unusable for viable CTC enrichment. In 2014, the same research group presented a 3D membrane microfilter with a separable bilayer design that did not require pre-fixation and therefore, was capable of capturing viable CTCs (Fig. 2b) (Zhou et al. 2014). The filter consists of two parylene layers with a 10-µm gap in between for holding captured cells. The top layer with 40-µm holes is aligned with the bottom layer containing hexagonally arranged 8-µm holes. Enrichment of tumor cells is accomplished by capturing relatively large tumor cells but with minimal effect on viability due to the reduced mechanical stress on the cells. The measured capture efficiencies were reported as 83 ± 3% and 78 ± 4% for MCF-7 and MDA-MB-231 cell lines, respectively. Moreover, spiked cell viabilities were reported as 74 ± 2% for MCF-7 and 71 ± 9% for MDA-MB-231 cancer cell lines.

Aside from membrane filters, microfluidic devices with built-in filters have also been used for CTC enrichment. Tan et al. developed a label-free microfluidic device, capable of isolating cancer cells from whole blood via an array of crescent-shaped isolation traps with an efficiency of at least 80% for breast and colon cancer cells (Fig. 2c) (Tan et al. 2009). Because the device is fabricated out of polydimethylsiloxane (PDMS) using soft lithography (McDonald and Whitesides

2002), it is optically transparent and biocompatible. The device exploits the size and deformability differences to isolate tumor cells from blood cells. Moreover, it preserves the viability and integrity of trapped tumor cells, which can also be retrieved by reversing the flow direction. Average recovery rates for different cell lines were reported to be 95, 97, and 96% for MCF-7, MDA-MB-231, and HT-29 cell lines, respectively. Importantly, the purity of isolated cells (>80%), one of the main obstacles in filtration-based technologies, has been shown to be comparable with some of the immunoaffinity-based enrichment technologies, which will be discussed in the following sections. Yoon et al. introduced a microfluidic chip filter that specifically addresses the clogging problem, which is common to most of the microfiltration-based CTC enrichment technologies (Yoon et al. 2016). Clog-free isolation of CTCs is achieved with an induced fluid oscillation by a piezoelectric actuator which displaces the cells trapped on the filter, thereby allowing the small cells to pass through the filter to be collected at the waste outlet (Fig. 2d). CTCs remaining on the filter are then retrieved by switching the fluid flow direction. The tests on the filter were conducted with 20-μm and 5-μm particles, representing CTCs and red blood cells (RBCs), respectively. Both separation efficiency and purity were reported as 100% while sieved particles were retrieved with 99.2% efficiency. Moreover, MDA-MB-231 tumor cells spiked into whole blood was processed without any clogging.

Microfiltration techniques are widely used in CTC enrichment due to their simple design, straightforward operation, and high processing throughput. However, filter-based CTC enrichment typically suffers from low specificity and purity due to the size overlap between CTCs and leukocytes (Ferreira et al. 2016; Ozkumur et al. 2013; Xu et al. 2015). In addition, filters are prone to clogging especially when processing large volumes of whole blood (Hosic et al. 2015; Ji et al. 2008).

2.2 Hydrodynamic Force-Based Enrichment Techniques

Another approach to enrich CTCs is to utilize unique hydrodynamics in microfluidic channels. Due to the small dimensions of a microfluidic channel, the flow is laminar (i.e., low Reynolds number) and fluids behave differently than macroscale turbulent flow that we are familiar with (Stone and Kim 2001). A popular technique that takes advantage of the laminar flow in microfluidic channels for size-based cell discrimination is the deterministic lateral displacement (DLD) (Holm et al. 2011; Huang et al. 2004; Inglis et al. 2006). In DLD, cells impinging on periodically shifted rows of micropillars are either deflected by pillars in one direction or flow through them undeflected depending on how the cell size compares with a set threshold (Fig. 3a). Since DLD is a continuous-flow process, it is less prone to clogging compared to microfilters (Yoon et al. 2016). Loutherback et al. have demonstrated the effectiveness of DLD arrays by successfully isolating large CTCs (i.e., 15–30 μm in diameter) (Meng et al. 2004) from blood cells, typically smaller than 15 μm (Loutherback et al. 2012). Moreover, DLD has been applied on

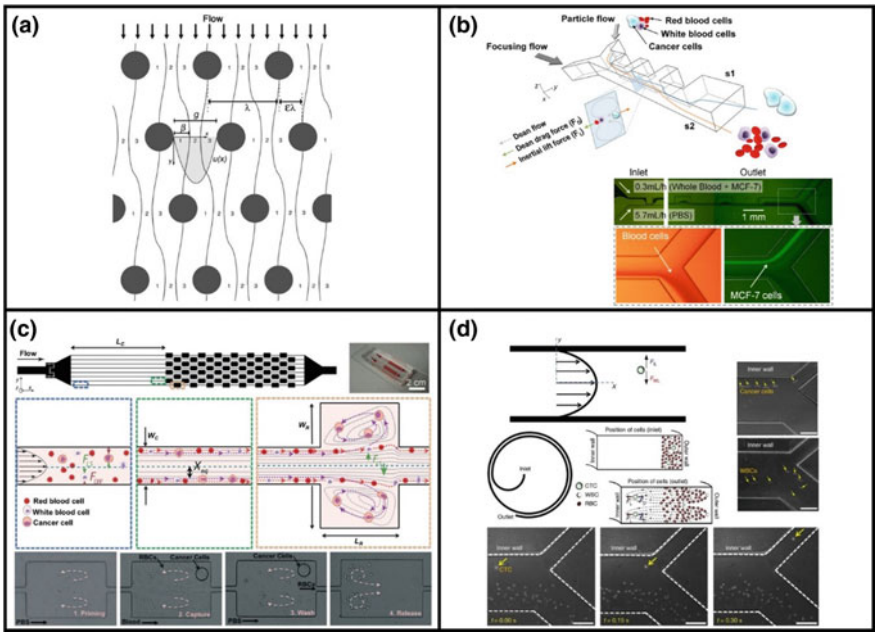

Fig. 3 **a** Schematic showing the principle behind the DLD operation. **b** Schematic drawing of the CEA chip and separation results from spiked cancer cells in whole blood. **c** Drawing of the microfluidic Vortex Chip operation with separated cells in each stage. **d** Working principle of the spiral microfluidic channel and cell separation results in time sequential order. Figure panels adapted and reproduced from Inglis et al. (2006) with permission from The Royal Society of Chemistry; Lee et al. (2013b) with permission from American Chemical Society; Sollier et al. (2014) with permission from The Royal Society of Chemistry; Warkiani et al. (2016) with permission from Nature Publishing Group

suspensions of particles with sizes ranging from a few nanometers up to tenth of micrometers (Davis et al. 2006; Wunsch et al. 2016) and have been proven to efficiently separate particles under various operating conditions. Loutherback et al. have also shown that the triangular pillar arrays are more effective than conventional circular pillar arrays in separating cells (Loutherback et al. 2010). The microfluidic device was tested by processing a diluted blood sample at a rate of 10 mL/min, and 86% of spiked cancer cells were recovered with negligible effects on cell viability (Loutherback et al. 2012). Despite requiring sample dilution, DLD is suitable for clinical studies because it can operate at high flow rates, which compensates the effect of dilution on the processing throughput.

Inertial focusing is another technique that exploits differences in biophysical properties of cells for the CTC enrichment. Inertial focusing effect was first demonstrated by Segre and Silberberg in the 1960s when it was observed that 1-mm-diameter suspended particles tend to move toward sidewalls of a 1-cm-diameter tube and form a ring (Segré 1961; Segré and Silberberg 1962). Only later, it was discovered that this phenomenon was due to inertial effects (Karnis

et al. 1966; Tachibana 1973). Lee et al. designed a microchannel device, called contraction–expansion array (CEA) (Fig. 3b), with alternating contraction and expansion regions to separate the cells under both the inertial lift force and the Dean drag force (Di Carlo et al. 2007; Lee et al. 2010, 2013b). Because the balance between the lift force and the Dean drag force depends on particle size, different-sized particles converge to different equilibrium positions in the microfluidic channel, thereby allowing cells to be separated from each other (Di Carlo 2009). Specifically, the larger cells or particles tend to be affected more by the inertial lift force and are pulled toward one side while smaller particles tend to be more affected by the Dean flow and are pulled toward the opposite side. It should be noted that the flow rate in the device is critical. For low flow rates, the inertial forces are negligible. For excessively high flow rates, the inertial forces dominate the Dean drag forces even for small particles, and therefore, the separation cannot be achieved. Under optimum conditions, the device was able to separate CTCs directly from non-diluted whole blood and achieve a CTC recovery rate of 99.1%, a blood cell rejection ratio of 88.8%, and a throughput of 1.1×10^8 cells/min as reported by Lee et al. (2013b). Moreover, it was found that by connecting two chips together, the blood cell rejection ratio of 97.4% could be achieved.

Vortex trapping is another hydrodynamics-based CTC enrichment technique (Hur et al. 2011). It operates by trapping target cells in the microvortices formed in the microfluidic device. Vortex Chip (Fig. 3c) introduced by Sollier et al. is similar to the CEA chip described previously in design except that it has symmetric expansion sections that produce microvortices (Sollier et al. 2014). The mathematics of vortex formation in these regions was also studied by Moffatt and Cherdron (Cherdron et al. 1978; Moffatt 1964). The Vortex Chip employs a long channel to first focus randomly distributed cells to their equilibrium lateral positions under the shear-gradient lift force and the wall-effect lift force (Hur et al. 2010). The shear-gradient lift force directs the cells toward the wall of the microfluidic channel while the wall-effect lift force pushes the cells toward the centerline of the channel. The shear-gradient lift force is stronger on larger cells; therefore, when the CTCs enter the vortex region, the wall-effect lift force is greatly reduced, driving larger CTCs deeper into the vortex region while leaving smaller cells such as RBCs and white blood cells (WBCs) to stay in the main channel. CTCs can later be retrieved by first washing the chip with PBS to remove the remaining cells in the channel, and then releasing the trapped CTCs by simply reducing the flow rate to allow the CTCs to escape from vortex regions. The Vortex Chip was tested with spiked cancer cells in whole blood. With the 20× diluted samples, on average 20.7% of the spiked cancer cells could be retrieved with a purity as high as 89%. Tests with patient samples also showed high purity ranging from 57 to 95%. It was also demonstrated that the Vortex Chip had negligible effect on cell viability.

Another CTC enrichment microfluidic device that employs inertial focusing is developed by Warkiani et al. (2016). In this device, microfluidic channel follows a circular pattern that leads to different equilibrium positions for CTCs and smaller blood cells based on the balance between the inertial lift and Dean drag forces. Specifically, CTCs are forced to move toward the inner wall of the device, while

hematologic cells such as WBCs, RBCs, and platelets follow the Dean vortices and move toward the outer wall (Fig. 3d) (Hou et al. 2013a). Using lysed blood samples spiked with different cancer cell lines, the device was shown to recover more than 85% of spiked cancer cells for each cell line with a median WBC contamination of 3109 WBCs per milliliter. Importantly, the device could deplete 99.99% of the WBCs from the lysed blood sample.

Hydrodynamic-based enrichment techniques have the advantage of label-free isolation of viable cells with relatively higher throughput without clogging problems; however, precise control over the sample flow rate and device geometry is a must for reliable CTC enrichment.

2.3 Acoustophoresis-Based Enrichment Techniques

Acoustophoresis-based CTC enrichment is a label-free method that relies on acoustic waves in the fluid to separate cells based on their biophysical properties and that can preserve the integrity and viability of cells. In acoustophoresis, cell movement is induced by standing acoustic pressure waves generated by a transducer (Fan and Vitha 2016). Standing acoustic waves in the microfluidic channel have nodes and anti-nodes, which are the points of minimum and maximum pressure oscillations. Due to pressure oscillations, cells experience forces proportional to their sizes. Moreover, the direction of the force depends on the acoustic contrast factor determined by the cell density and compressibility relative to the medium. Cells with positive acoustic contrast factor move toward the nodes while cells with negative acoustic contrast factor move toward the anti-nodes. In acoustophoresis-based CTC enrichment techniques, both the pace and direction of migration can be used to separate cells based on the contrast in their biophysical properties.

Antfolk et al. reported a silicon micromachined microfluidic chip that employed acoustophoresis for the separation of CTCs from WBCs (Antfolk et al. 2015). The chip comprises two channels, a pre-alignment channel and a separation channel, that are actuated by two piezoelectric transducers operated at 4.530 and 2.001 MHz, respectively. In the pre-alignment channel, cells are pushed toward pressure nodes near the sidewalls of the microfluidic channel, replacing otherwise needed hydrodynamic pre-focusing of cells before the separation process. In the separation channel, CTCs are selectively moved toward the pressure node at the center under higher acoustic forces, while smaller leukocytes remain closer to the walls (Fig. 4a). By collecting laterally separated cells from two different outlets, Antfolk et al. reported a CTC recovery rate of $86.5 \pm 6.7\%$ with $1.1 \pm 2.8\%$ contamination for prostate cancer cells (DU145) spiked into a RBC-lysed whole blood sample. Increasing acoustic field intensity led to a higher recovery rate of $94.8 \pm 2.8\%$ at the expense of higher contamination of $2.2 \pm 0.6\%$.

While acoustophoresis-based separation of CTCs from WBCs has been demonstrated by different research groups (Antfolk et al 2015; Augustsson et al. 2012; Ding et al. 2014), long-term operational instability and low throughput of

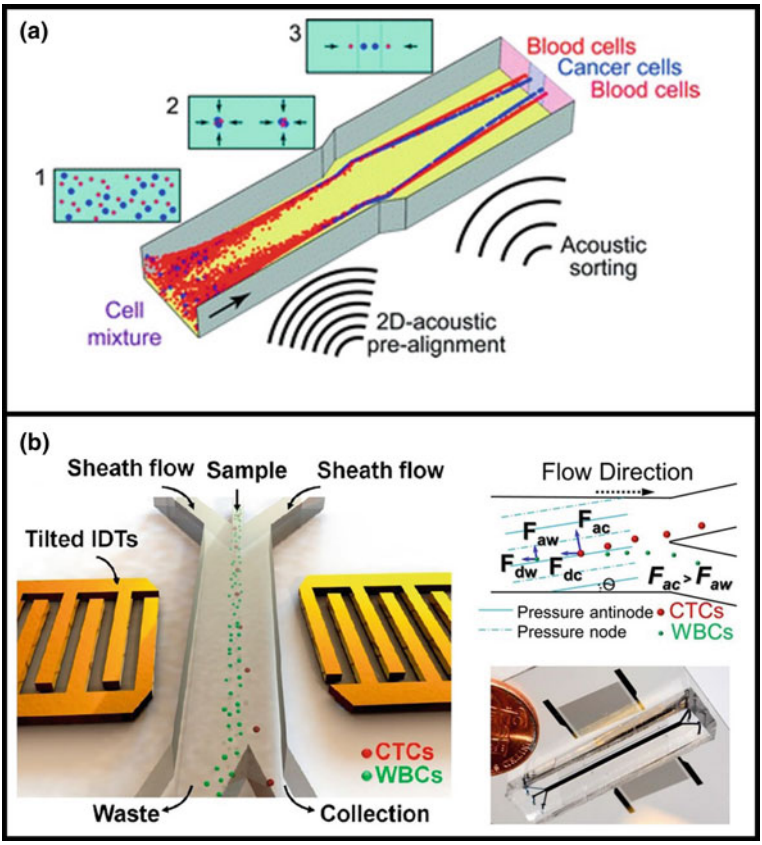

Fig. 4 **a** Representation of two-stage acoustophoresis chip with illustrative cross-sectional cell positions during the separation process. **b** Schematic illustration and actual view of taSSAW device. Figure panels adapted and reproduced from Antfolk et al. (2015) with permission from Creative Commons; Li et al. (2015) with permission from the National Academy of Sciences

these devices have limited their use on clinical samples. In 2015, Li et al. achieved enrichment of breast cancer patient CTCs by using tilted-angle standing surface acoustic waves (taSSAW) device (Fig. 4b) (Li et al. 2015). In this device, tilted interdigitated transducers (IDTs) are used as actuators, forming multiple regions of slanted nodes and anti-nodes inside the microfluidic channel. As cells pass through these regions, they experience different levels of acoustic forces, which manipulate their positions inside the channel. At a flow rate of 1.2 mL/h, experiments using cell lines spiked into RBC-lysed blood samples showed recovery rates greater than 87% for MCF-7 and HeLa, and 83% for UACC-903 M and LNCaP cancer cell lines. The device was also tested on clinical samples collected from three breast cancer patients. For the first two patients, 59 and 8 CTCs were detected from 2 mL of

blood samples. For the third patient, only one CTC was detected within 6 mL of blood sample.

While gentle and label-free characteristics of acoustophoresis-based enrichment techniques make them well suited for isolation of viable CTCs, low throughput currently limits their use in clinical settings.

2.4 Dielectrophoresis-Based Enrichment Techniques

Apart from size, density, and deformability, electrical properties of CTCs can also be used for discriminating them from other components of blood. In dielectrophoresis (DEP)-based enrichment techniques, non-uniform electric field and polarization of cells are used for attraction or repulsion, depending on dielectric constant. If the cells move toward the source of the electric field, it is called positive DEP (pDEP); however, if the cells move away from the higher electric field, it is called negative DEP (nDEP). By setting the electrical excitation frequency in between cross-over frequencies for different cells, it is possible to separate different cell populations by driving them in opposite directions.

Commercial ApoStream™ (ApoCell) system utilizes dielectrophoretic field-flow fractionation (DEP-FFF) for label-free isolation of viable cancer cells (Fig. 5a) (Gupta et al. 2012). This system employs a 45–65 kHz AC signal, which is in between the cross-over frequencies of cancer cells (around 30–40 kHz) and peripheral blood cells (90–140 kHz) for attracting cancer cells toward electrodes and repelling blood cells to the center of the channel. Enriched cancer cells are collected through the product collection port while excluded blood cells exit the chamber through the waste outlet. In experiments using two different cell lines, SKOV3 and MDA-MB-231 cells, the recovery rates were reported as 75.4 ± 3.1% and 71.2 ± 1.6%, respectively. Moreover, the viability of enriched cancer cells was greater than 97.1%. By using the advantage of the continuous-flow design, ApoStream™ is able to process 7.5 mL sample within 1 h, higher than most dielectrophoresis-based CTC enrichment techniques (Cheng et al. 2007; Kuczenski et al. 2011).

Apart from different planar designs, Cheng et al. used 3D lateral dielectrophoresis for CTC enrichment (Cheng et al. 2015). In this device, a 3D, V-shaped serpentine microchannel, having an electric field between the top surface and bottom slanted sidewalls, is utilized for DEP-based separation of cancer cells (Fig. 5b). Cells, hydrodynamically focused near channel sidewalls, are exposed to DEP force throughout the channel, separating tumor cells by forcing them to migrate toward the center of microchannel. The device was tested with AS2-GFP lung cancer cells for a flow rate of 20 μL/min, and 85% recovery rate was achieved with a purity of 90%. Increasing the flow rate to 2.4 mL/h resulted a recovery rate of 81%.

Making use of the intrinsic electrical properties of CTCs to identify them without any labeling and the ability to isolate single tumor cells with high viability make dielectrophoresis-based isolation techniques attractive. However, processing

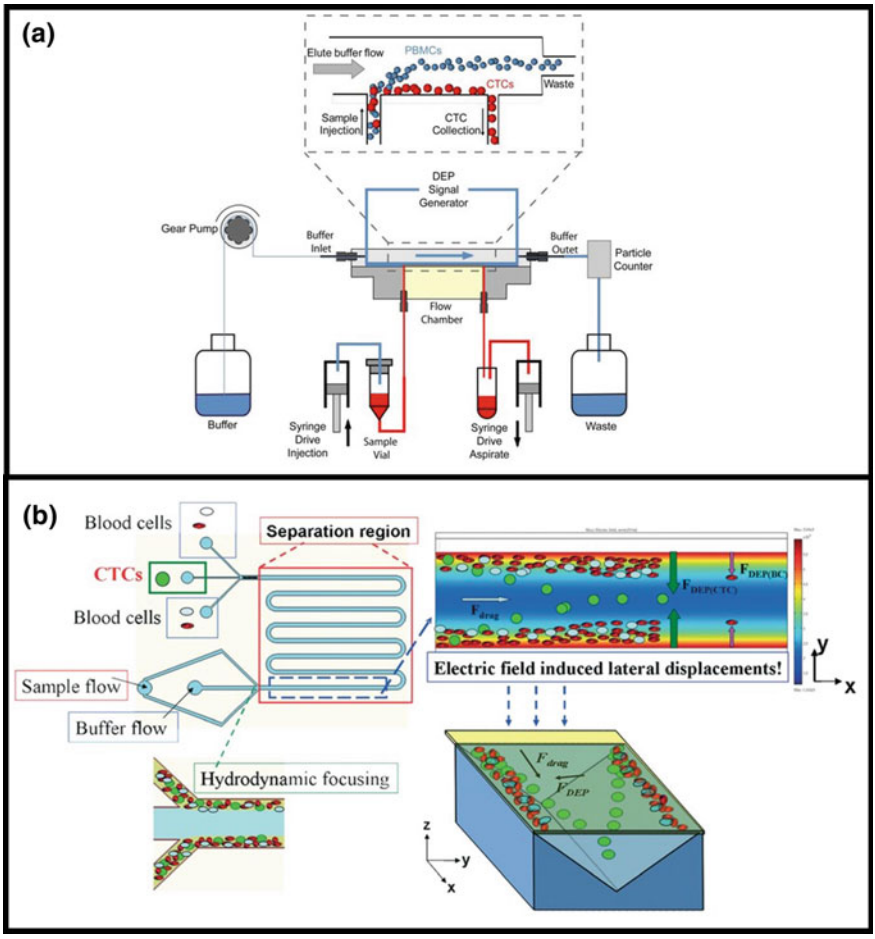

Fig. 5 **a** Schematic drawing of ApoStream system operation. **b** Illustration of designed microfluidic chip and visualized dielectrophoretic forces inside the V-shaped channel. Figure panels adapted and reproduced from Cheng et al. (2015) with permission from The Royal Society of Chemistry; Gupta et al. (2012) with permission from AIP Publishing

throughput and sample purity are among the challenges that need to be addressed before DEP-based CTC enrichment technologies can be used for clinical applications.

2.5 CTC-Cluster Enrichment Techniques

Besides single tumor cells in circulation, aggregates of CTCs, referred as CTC-clusters or circulating tumor microemboli (CTM), also contribute to cancer metastasis. Although CTC-clusters are extremely rare, constituting only 2–5% of all

CTCs, studies on animal models showed greater (as high as 50 times) metastatic propensity of tumor cell clusters than individual CTCs (Aceto et al. 2014; Watanabe 1954). While CTC-clusters can also serve as biomarkers for early detection of cancer as well as providing crucial information on the course of treatment (Goto et al. 2017; Hong et al. 2016), detailed studies on this important CTC subpopulation have been hindered by the lack of optimized technologies to isolate CTC-clusters from clinical samples. Although CTC-clusters are detected with some of the technologies developed for single CTCs, the sensitivity and specificity of these technologies are low (Hou et al. 2013b; Stott et al. 2010; Vona et al. 2000). Moreover, high shear stress on clusters risks dissociating them into single cells and creating artifacts in the enrichment process. To further understand clusters and their potential implications for metastatic cancer, efficient and reliable enrichment of viable clusters is essential.

The first device specifically targeting CTC-clusters, the Cluster-Chip, was developed by Sarioglu et al. (Figure 6) (Sarioglu et al. 2015). The Cluster-Chip isolates CTC-clusters from unprocessed whole blood without any labeling for tumor-specific markers, making it applicable to different types of tumor cells independent of their surface antigens. The chip exploits the geometry of clusters

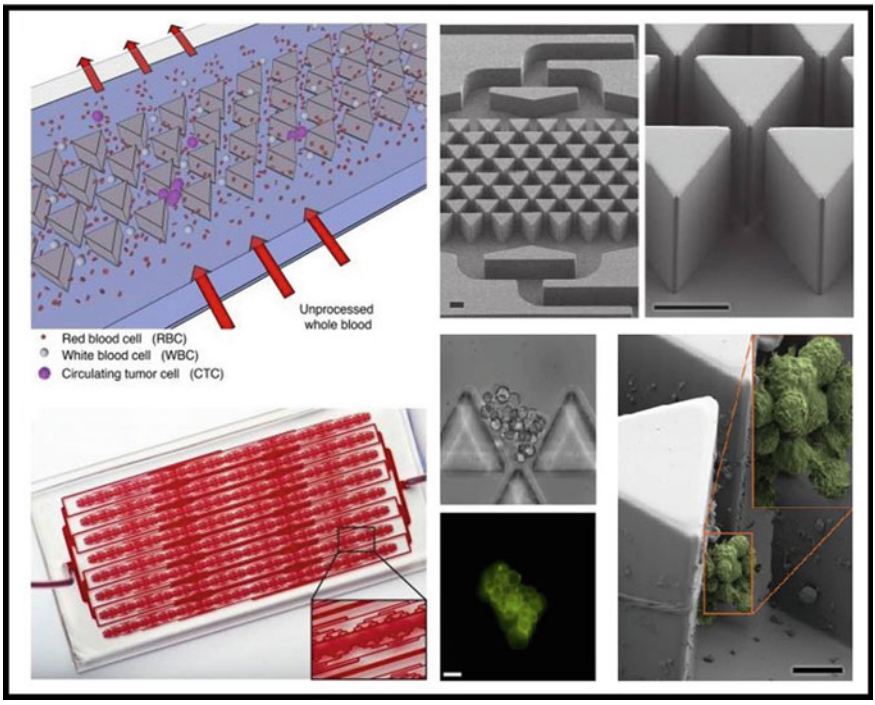

Fig. 6 Design and operational illustration of Cluster-Chip with SEM & fluorescent microscope images of captured CTC-clusters. Figure panels adapted and reproduced from Sarioglu et al. (2015) with permission from Nature Publishing Group

and strength of cell-cell junctions. The device employs specialized cluster traps made up of three triangular pillars. Among the three, two of the triangular pillars form narrowing channel that guides the cells to the bifurcation nodes, and the edge of the third pillar bifurcates laminar flow to divert cells through one of the two streamlines through 12 × 100 μm openings. While single cells have to commit to one of the openings, CTC-clusters are retained under a dynamic force balance due to simultaneous interaction with both streamlines. The device operates at sub-physiological flow rates, which are significantly lower than microfiltration-based enrichment techniques, resulting in low shear stress microenvironment that preserves the integrity of clusters. Unlike microfiltration-based approaches, clogging is not a problem since the single cells are not trapped by the Cluster-Chip. Therefore, high volume of whole blood can be processed without any need for sample manipulation. With the flow speed of 2.5 mL/h, Cluster-Chip captured 169/171 (99%) MDA-MB-231 clusters of at least four cells, 28/40 (70%) of three-cell clusters, and 48/117 (41%) of two-cell clusters. Release of captured CTC-clusters is done on a thermoelectric cooler at 4 °C to reduce the non-specific cell adhesion under reverse flow. Under these conditions, 188/236 (80%) of the captured clusters were released without a prominent effect on cell viability. Cluster-Chip technology was applied to clinical samples collected from 60 patients with metastatic melanoma, breast and prostate cancers, and identified CTC-clusters in 40% of these patients. The efficient and gentle nature of the Cluster-Chip also enabled single-cell RNA sequencing to be performed on isolated CTC-clusters as wells as on WBCs traveling attached to them. Moreover, patient CTC-clusters enriched by the Cluster-Chip were later used for biophysical studies on CTC-cluster circulation that broke the misconception of clusters being too large to pass through the narrow capillaries by showing that CTC-clusters can reorganize as single-file chains and can pass through narrow capillary-sized vessels (Au et al. 2016).

Recently, Au et al. introduced a two-stage continuous-flow microfluidic chip designed to isolate viable CTC-clusters by exploiting the size and asymmetrical properties of them (Fig. 7) (Au et al. 2017). The chip utilizes the modified version of deterministic lateral displacement (DLD) technique. The first stage is similar to the conventional DLD device (Huang et al. 2004; Inglis et al. 2006), having polydimethylsiloxane (PDMS) pillars and was used for deflecting large clusters. The second stage, which receives the undeflected components in stage 1 (i.e., single cells and small clusters), comprises asymmetrically shaped pillars for deflection of small clusters. This stage has 30-μm-height channel for constraining and aligning the longitudinal axes of clusters to the X-Y plane. Unlike the first stage, asymmetry of clusters in addition to size is used in this stage. Selected flow rate of 0.5 mL/h resulted in shear stresses of 2.9 and 4.8 Pa, lower than the shear stress in human vasculature (Chandran et al. 2007), leading to recovered cell viabilities over 87%. The recovery rates for cells spiked into buffer were calculated as 99.3 ± 1.1% for large clusters and 79 ± 6.1% for small clusters. Recovery rates for the cluster of CTCs residing in whole blood decreased to 98.7 ± 2.4% for large clusters and 65.5 ± 6.5% for smaller ones.

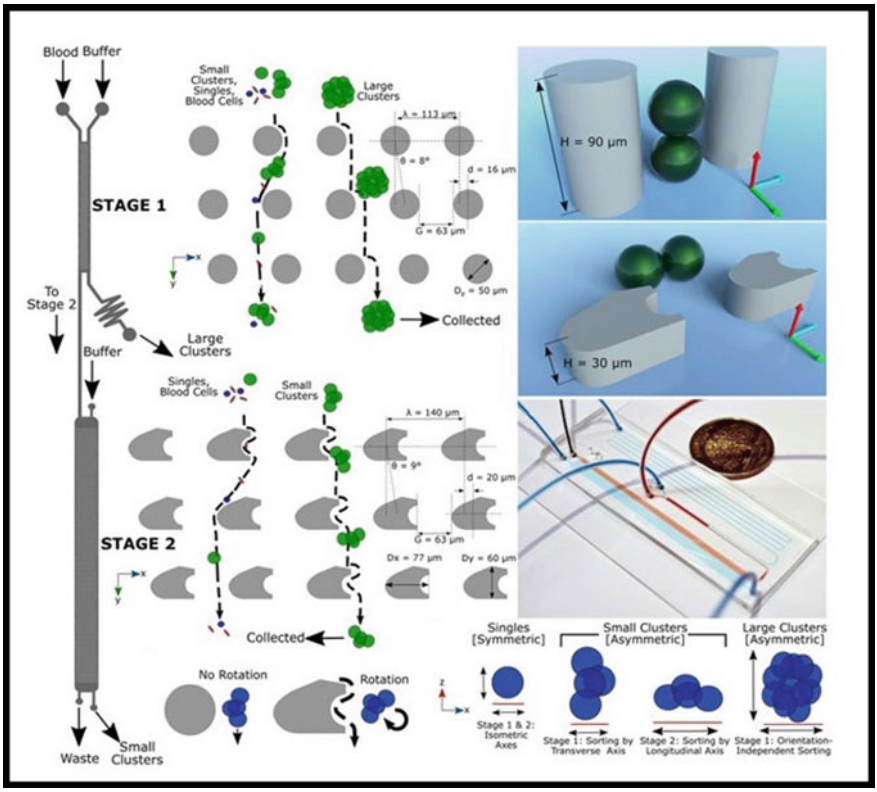

Fig. 7 Operation principle of two-stage microfluidic chip designed to enrich CTC-clusters. Figure panels adapted and reproduced from Au et al. (2017) with permission from Creative Commons

Recent advances in the development of optimized technologies to enrich viable CTC-clusters are important as higher specificity and sensitivity provided by these technologies will be instrumental in enabling clinical and basic research to understand their clinical utility and physiological role in cancer metastasis.

3 Biochemical Contrast-Based Enrichment of CTCs

CTC enrichment based on biophysical properties can achieve high-throughput, label-free enrichment, but faces challenges in improving the specificity, and hence, the purity of enriched CTCs (Song et al. 2017; Yu et al. 2011). Biochemical targeting of CTCs relies on the highly specific interaction between ligands and tumor-specific antigens present on the cell membrane of CTCs. In most approaches, affinity ligands (antibodies or aptamers) are immobilized on either a microdevice or

magnetic beads with enhanced surface-to-volume ratio to achieve high capture efficiency with high purity (Hoshino et al. 2011; Lee et al. 2013a). Most immunoaffinity methods use epithelial cell adhesion molecule (EpCAM) as the biomarker, which is commonly expressed on CTCs of epithelial cancers, such as cancers of lung, liver, breast, prostate, and colon (Went et al. 2004), or a tissue specific membrane protein such as epidermal growth factor receptor 2 (HER2) for breast cancer or prostate specific membrane protein (PSMA) for prostate carcinoma (Alix-Panabières and Pantel 2014).

This subsection will focus on the technologies exploiting the differences in cell membrane proteins between CTCs and blood cells for enrichment.

3.1 Antibody-Based CTC Enrichment

The contrast in the expression levels of specific cell membrane antigens between tumor cells and blood cells is used for immunoaffinity-based CTC enrichment methods (Pantel et al. 2008). Most of the antibody-based CTC enrichment techniques use antibody conjugated devices or magnetic beads and target epithelial biomarkers (Saliba et al. 2010). EpCAM is the most commonly used biomarker to directly capture epithelial CTCs (Allan and Keeney 2009; Stott et al. 2010; Talasaz et al. 2009), while CD45 is the most commonly targeted biomarker to deplete the leukocytes (Giordano et al. 2012; Lara et al. 2006).

Immunomagnetic CTC enrichment has been the most widely used method wherein magnetic beads coated with antibodies bind to cells to isolate CTCs from blood samples in an external magnetic field. In the CellSearch® system (Pantel et al. 2008; Riethdorf et al. 2007), EpCAM-positive tumor cells are separated from blood cells using EpCAM-specific antibodies conjugated to ferrofluid magnetic particles and then sequentially fixed and stained with fluorescent anti-cytokeratin and DAPI (4,6-diamidino-2-phenylindole), while hematopoietic cells are stained with anti-CD45 antibodies. Finally, cells that are positive for cytokeratin and DAPI, and negative for CD45, are identified as CTCs and counted using a semi-automated fluorescent microscope. CellSearch® system is limited to capture CTCs that only express EpCAM and cytokeratins (CKs). There is a 20–40% cell loss because of the inability of detecting cancer cells with a reduced EpCAM expression that have gone through epithelial–mesenchymal transition (EMT) (Alix-Panabières and Pantel 2013). Magnetic activated cell sorting (MACS) system is another popular technology that can be used for positive and negative CTC enrichment. In this system, epithelial cancer cells are enriched from blood by incubation with anti-EpCAM antibody-coated ferromagnetic microbeads, followed by magnetic separation using a column in a strong magnetic field (Miltenyi et al. 1990). Using this technology, 10^9 cells could be sorted in 15 min with >100-fold enrichment and with 90% viability. Magnetic labeled CTCs expressing EpCAM stay within the column and are then recovered by removing the magnetic field. Dynabeads magnetic separation technology is a similar technology to MACS but does not require column for magnetic separation (Neurauter et al. 2007). Magnets in this system are designed to

separate magnetically labeled cells right away without further steps. In general, the limitation of this technology is the inconsistent cell recovery rates (Lin et al. 2011).

The first immunoaffinity-based microfluidic technology for CTC enrichment, the CTC-chip, was developed by Nagrath et al. (2007). The system was able to isolate CTCs with high sensitivity and specificity from whole blood. CTC-chip was microfabricated out of silicon using deep reactive-ion etching (DRIE) and consisted of an array of 100-μm-tall microposts functionalized with the anti-EpCAM antibody (Fig. 8a). With the geometric arrangement of 78,000 microposts optimized for efficient cell-micropost interaction, CTCs were detected in 115 of 116 cancer patients (79–196 CTCs/mL) while no CTCs were found in control samples from healthy individuals. Following the development of the CTC-chip, numerous EpCAM-based microfluidic CTC enrichment technologies with varying degrees of sensitivity and purity were developed over the last decade (Chen et al. 2011; Choi et al. 2013; Davies et al. 1994; Harb et al. 2013; Hughes and King 2010; Kim et al. 2014; Lu et al. 2010b; Mittal et al. 2012; Nagrath et al. 2007; Ozkumur et al. 2013; Wang et al. 2009, 2011). Herringbone (HB) chip operated on the same basis as the CTC-chip except that it was made out of PDMS and included herringbone chevrons rather than microposts (Stroock et al. 2002). The HB chip was designed to enhance CTC capture efficiency through the herringbones that disrupted the laminar flow by inducing microvortices, which increased the interactions of CTCs with the functionalized surfaces in the device (Stott et al. 2010). The HB chip showed a >90% capture efficiency for spiked PC3 cells, compared to a ~68% capture efficiency of CTC-chip at the same flow rate (1 mL/h). The device was also tested on patients with metastatic prostate cancer and CTCs were detected in 14 of the 15 patients. The NanoVelcro CTC-chip employs nanopillar coatings that maximize the frequency of contact between CTCs and anti-EpCAM antibodies to increase the CTC capture rate (Lu et al. 2013). A capture efficiency greater than 80% was reported for LNCaP, PC3, C4-2 prostate cancer cell lines in both PBS and blood. High-throughput microsampling unit (HTMSU), which is an electrokinetic enrichment device, has 51 sinusoidal microchannels (35 μm width × 150 μm depth) coated with anti-EpCAM antibodies and captured CTCs from whole blood with a reported 97% cell recovery (Adams et al. 2008). Recently, a 3D scaffold microchip has been developed to separate single and cluster of CTCs from the whole blood (Fig. 8b). The 3D scaffold chip with interconnected macropore structure, which significantly increases the contact frequency between cells and immobilized anti-EpCAM antibodies, improves the CTC capture efficiency even at high flow rate from whole blood (Cheng et al. 2016). The device successfully captured 1–118 CTCs/mL from 14 cancer patients, while 1–14 CTC-clusters/mL were detected from 5 out of these 14 cancer patients at a 100 μL/min flow rate. Immunomagnetic CTC isolation with an integrated high-throughput device is also a popular technology that has been widely used for CTC enrichment from the whole blood. Besant and Poudineh et al. developed a microfluidic device patterned with X-shaped microstructures that separated magnetically labeled CTCs in different regions of the device (Fig. 8c). By controlling the velocity or magnetic field strength, high EpCAM-expressing cells with high nanoparticle loading were

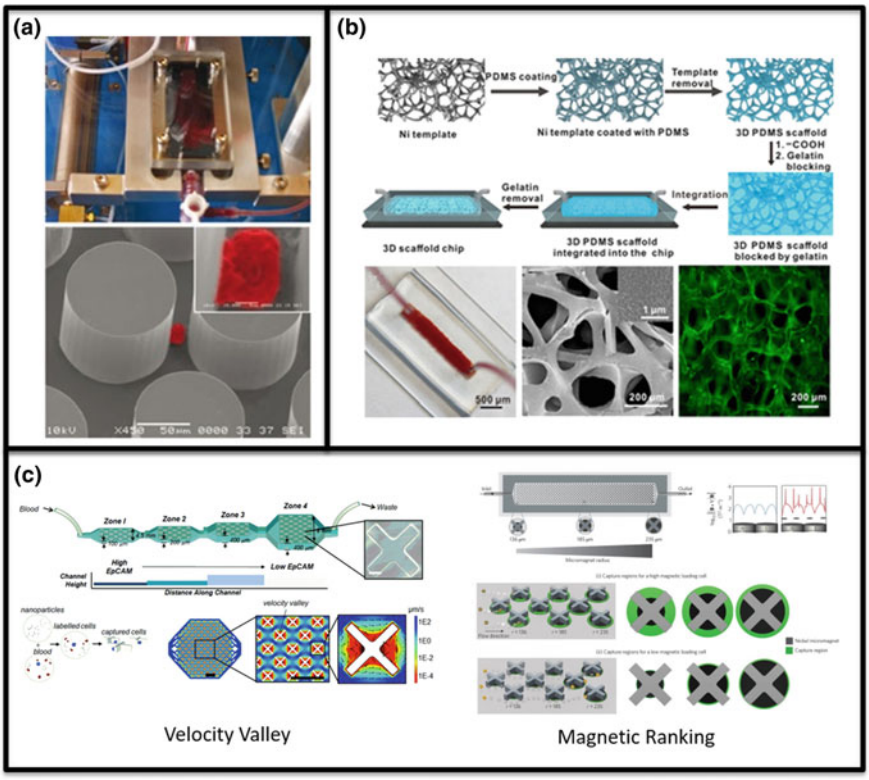

Fig. 8 Design and working principles of representative antibody-based CTC enrichment technologies **a** CTC chip, **b** 3D PDMS scaffold chip, **c** microfluidic technology for magnetic phenotypic ranking of CTCs. Figure panels adapted and reproduced from Besant et al. (2015) with permission from The Royal Society of Chemistry; Cheng et al. (2016) with permission from American Chemical Society; Nagrath et al. (2007) with permission from Nature Publishing Group; Poudineh et al. (2017a) with permission from Nature Publishing Group

captured in different regions than low EpCAM-expression cells with less nanoparticle loading, which provided phenotypic ranking of CTC antigen expression with >90% recovery (Besant et al. 2015; Poudineh et al. 2017a).

Antibodies are widely used in CTC enrichment techniques due to their high specificity in identifying target cells, while limitations including the poor reproducibility, limited shelf life, high cost, and complex release process still remain as technical challenges for the antibody-based CTC enrichment (Jackson et al. 2017).

3.2 Aptamer-Based CTC Enrichment

Chemical antibodies, also known as aptamers, have been used as a replacement for the biological antibodies in CTC enrichment technologies due to its capability to

specifically bind to the target cells through affinity (Jackson et al. 2017; Nagrath et al. 2007; Phillips et al. 2008; Zhou and Rossi 2014). Aptamers are short single-stranded oligonucleotides that can recognize and specifically bind to their target cells by folding into unique 3D structural conformations (Sun et al. 2014). They can be easily generated using an in vitro selection process termed Cell-SELEX (systematic evolution of ligands by exponential enrichment) to target CTCs (Tuerk and Gold 1990). Recently, a tissue slide-based SELEX strategy has been used to produce high-affinity aptamers for heterogenic lung cancer cell markers from individual patient. This unique study shows the possibility of designing "personalized" aptamers to target tumor-specific markers in different patients (Zamay et al. 2015).

Aptamers offer many advantages, including high affinity, reproducibility, long shelf life, small size, lower cost, easy incorporation of diverse functional groups, and release mechanisms without damage to target cells (Bruno 2015; Bunka and Stockley 2006; Dickey and Giangrande 2016; Farokhzad et al. 2004; Song et al. 2012). While only a few antibodies have been identified to capture CTCs, a number of aptamers have been created to target cancer cells with high affinity and selectivity via cell-surface biomarkers (Dharmasiri et al. 2009; Qian et al. 2015). In addition, multiple diversified aptamers can efficiently recognize and distinguish CTCs based on the heterogeneous expression of surface markers (Poudineh et al. 2017b; Shen et al. 2013). Moreover, following the affinity selection, nuclease enzymes or complementary oligonucleotides can be used to cleave the aptamer link to release the tumor cells while maintaining their viability for further downstream analysis (Ma et al. 2015; Zao et al. 2017).

Liu et al. designed an aptamer–nanoparticle strip biosensor within a lateral flow device to detect and enrich cancer cells (Liu et al. 2009). In this study, the Cell-SELEX technique was used to identify two different aptamers (a thiolated aptamer TD05 and a biotinylated aptamer TE02) that are specific to Ramos cells. These two aptamers were then immobilized on gold nanoparticles and in the test zone of a nitrocellulose membrane, respectively. When Ramos cells interact with aptamer-functionalized gold nanoparticles on the lateral flow strip, gold nanoparticles accumulated in the test zone and produced a visible red band. However, this technique has a limitation on the blood sample volume due to the non-specific binding of erythrocytes on the membrane. Viraka Nellore et al. developed an RNA-aptamer-coated graphene oxide membrane with 20–40-μm-diameter pores to capture and identify multiple types of cancer cells from blood samples (Viraka Nellore et al. 2015). Aptamers were capable of capturing tumor cells SKBR3, LNCaP, and SW-948 with a 95% capture efficiency. In this study, different fluorescent-labeled aptamers were also utilized to identify different cell types using multicolor fluorescence imaging.

Microdevices with high-affinity aptamers have provided unique opportunities for CTC enrichment from patient blood samples (Myung and Hong 2015). For example, an aptamer-modified cell affinity chromatography-based microdevice has been demonstrated (Phillips et al. 2008). The device was capable of capturing target cells from a mixture of control cells with 97% purity and could simultaneously sort

cells into independent fractions with a 135-fold enrichment in a single run (Fig. 9a) (Xu et al. 2009). Zhao et al. developed a long multivalent DNA aptamer-based microfluidic device that employed rolling circle amplification method to capture the lymphoblast CCRF-CEM cells (Zhao et al. 2008). In this device, DNA aptamers, designed to bind to protein tyrosine kinase with multiple binding sites, formed a 3D network on the herringbone-patterned surface. This approach was demonstrated to yield a higher capture efficiency and purity of target cells than monovalent aptamers and antibodies under varying sample flow rates (0.06–6 mL/h) (Zhao et al. 2012). Another microfluidic device for aptamer-based capture of CTCs was introduced by Sheng et al. This microfluidic device with more than 59,000 micropillars was used to isolate as few as 10 tumor cells from a 1 mL of unprocessed whole blood sample with a >95% capture efficiency and high cell viability within 28 min (Sheng et al. 2012) (Fig. 9b). Similarly, a PDMS Hele-Shaw device was created with an array of pits which held glass beads functionalized with RNA aptamers. The device captured hGBM cells that overexpressed EGFR with 44% efficiency from a mixture of cells. Captured cells could then be released from the glass beads for downstream analysis with a 92% efficiency using complementary aptamers (Wan et al. 2012). Another RNA–aptamer biochip, a PDMS chip employing a microelectrode matrix on a silicon dioxide layer, was developed for capturing and detecting captured cells (Wang et al. 2012). In this device, the binding of tumor cells to anti-EGFR RNA aptamers could be detected due to the increase in the resistance between electrodes separated by a 2.5-μm gap.

Sheng et al. combined aptamer-functionalized gold nanoparticles with microfluidics to design a device with a coating of multivalent DNA aptamer nanospheres to capture human acute lymphoblastic leukemia (CEM) cells from whole blood efficiently (Fig. 9c) (Sheng et al. 2013). By using the gold nanoparticle–aptamer complex, the capture efficiency of CEM cells in buffer was increased from the 49% achieved using the aptamer alone to 92%. Integration of a herringbone structure in microfluidic device further improved the capture efficiency of CEM cells in whole blood from less than 60 to >90%. Recently, Zhao et al. have developed a microfluidic CTC-chip based on aptamer cocktails with synergistic effect (Zhao et al. 2016). With a single aptamer grafted on silicon nanowires, the CTC capture performance of the device was relatively weak (Fig. 9d). However, using aptamer cocktails led to not only higher capture affinity due to synergistic interaction between different aptamers but also a differential capture efficiency for different CTC subpopulations. With different combinations of aptamers, a >50% capture efficiency was achieved for certain non-small cell lung cancer (NSCLC) cell lines. Also, CTCs were successfully detected by aptamer cocktails from samples collected from 11 NSCLC patients. Based on these results, this method has the potential to provide more comprehensive information in treatment monitoring. Labib et al. had recently developed a new aptamer-mediated, 2D microfluidic assay to sort CTCs according to different expression levels of surface markers. In this device, cancer cells labeled with aptamer conjugated magnetic nanoparticles are separated in a two-stage process into 16 different subpopulations based on their expressions of EpCAM and HER2 (Labib et al. 2016).

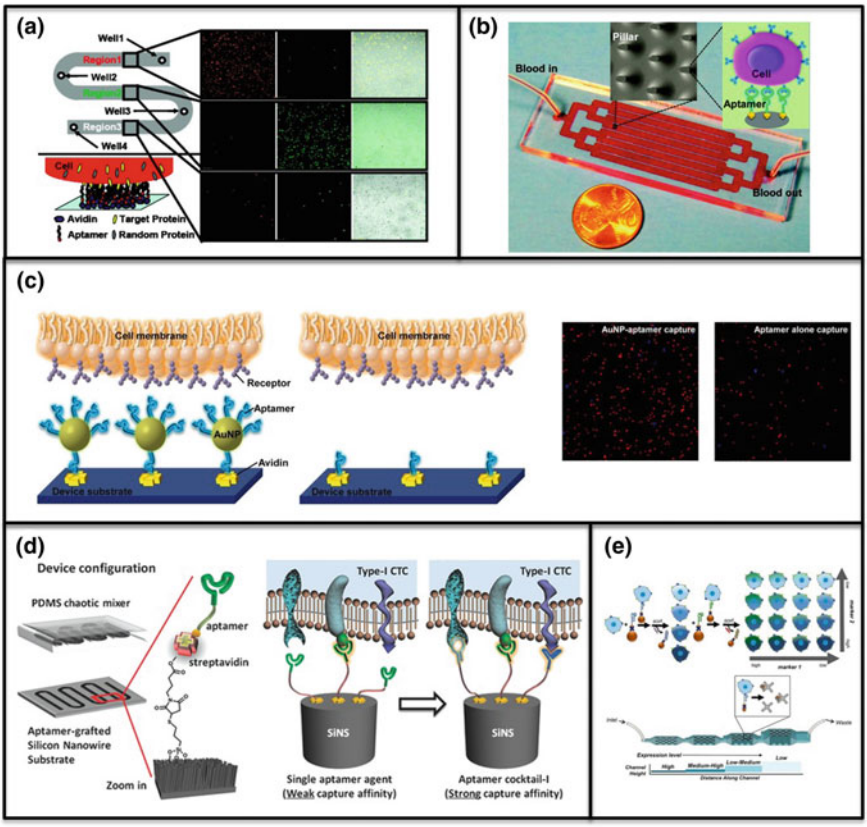

Fig. 9 Design and working principles of representative aptamer based CTC enrichment technologies. **a** Aptamer-functionalized microfluidic device with multiplexed detection. **b** Micropillar modified microfluidic device coated with DNA aptamers. **c** Aptamer cocktail modified microfluidic CTC chip. **d** AuNP-aptamer modified microfluidic device. **e** 2D sorting aptamer microfluidic device. Figure panels adapted and reproduced from Labib et al. (2016) with permission from American Chemical Society; Sheng et al. (2012) with permission from American Chemical Society; Sheng et al. (2013) with permission from American Chemical Society; Xu et al. (2009) with permission from American Chemical Society; Zhao et al. (2016) with permission from Wiley

Early studies on aptamer-based CTC enrichment platforms show great improvement of the sensitivity especially when multiple aptamers are used to capture CTCs. Although antibodies are more common as targeting ligands in the current CTC enrichment technologies, unique advantages offered by aptamers make them viable candidates to be used in technology development for CTC enrichment.

4 Hybrid Technologies

CTC enrichment technologies that combine different biophysical and/or biochemical principles have also been introduced. As explained in the previous sections, each technique has its own advantages and limitations, so combining them in the same device can mitigate the drawbacks faced by each alone and provide higher sensitivity and specificity for CTC enrichment. In the CTC-iChip developed by Ozkumur et al. and Karabacak et al., deterministic lateral displacement (i.e., size-based isolation), inertial focusing and affinity-based negative selection through magnetophoresis are combined in one device (Ozkumur et al. 2013; Karabacak et al. 2014). In the negative selection mode (^{neg}CTC-iChip), the whole blood sample is pre-mixed with CD15 and CD45 immunomagnetic beads to label WBCs (Fig. 10a). In the ^{pos}CTC-iChip, CTCs are pre-labeled with anti-EpCAM conjugated immunomagnetic beads for positive selection. In operation, first smaller RBCs platelets are eliminated by the DLD stage, leaving the nucleated cells (WBCs and CTCs) for the next stage. The WBCs and CTCs were then focused into a single-file arrangement by inertial focusing as they passed through a serpentine channel. Lastly, the CTCs and tagged WBCs were introduced to an external magnetic field where magnetically labeled cells, WBCs for ^{neg}CTC-iChip and CTCs for ^{pos}CTC-iChip, were deflected to achieve CTC enrichment. The recovery rate of the ^{pos}CTC-iChip for different cell lines with varying levels of EpCAM expression ranged from 98.6% (SKBR3) to 77.8% (MDA-MB-231) whereas the

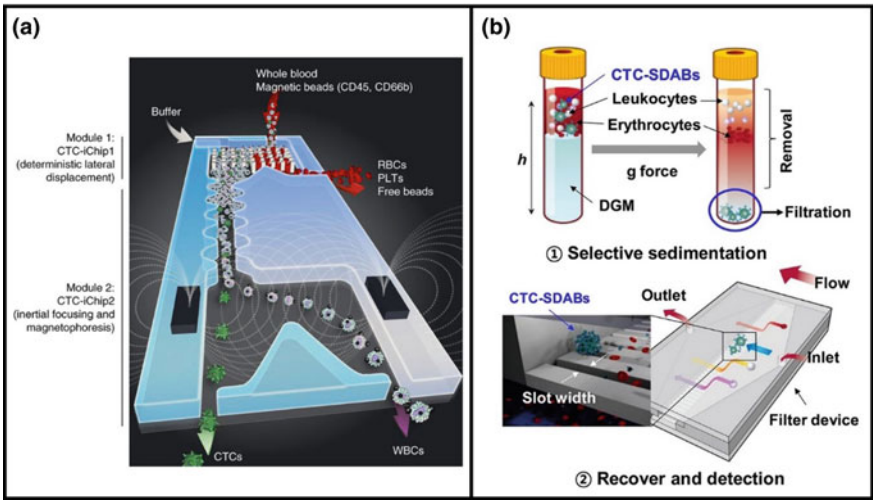

Fig. 10 **a** Schematic of CTC-iChip is illustrated with magnetic beads attached to WBCs for negative depletion. **b** Working principle of the CTC enrichment process that combines density gradient centrifugation and a microfiltration device. Figure panels adapted and reproduced from Karabacak et al. (2014) with permission from Nature Publishing Group; Park et al. (2012) with permission from American Chemical Society

recovery rate of the ^{neg}CTC-iChip was fairly constant at about 97% for both cell lines (MCF10A and MCF10A-LBX1) tested. The performance of the ^{pos}CTC-iChip was also compared with the CellSearch® system and was found that when the CTCs count was lower than 30 CTCs per 7.5 mL, the performance of the ^{pos}CTC-iChip was significantly better than the CellSearch® system in terms of recovery rate.

Some of the CTC enrichment technologies combine pre-processing of samples using batch processes with microfluidic systems to achieve higher recovery ratio and purity for CTCs. Park et al. combined immunoaffinity, density gradient centrifugation, and on-chip microfiltration to enrich spiked breast cancer (MCF-7) and lung cancer (DMS-79) cells in whole blood (Fig. 10b) (Park et al. 2012). The motivation of this technology is to increase the recovery rate of size-based CTC enrichment techniques that otherwise suffer from WBC contamination due to the size overlap between CTCs and WBCs. In this work, size–density amplification beads (SDABs) are first attached to CTC membrane through immunoaffinity. Labeled CTCs can then be separated from the rest of the blood cells through density gradient centrifugation. Due to the attachment of SDABs, the CTCs aggregate at the bottom layer of the tube, making the aspiration of other blood cells less lossy. Residual cells were first resuspended and then passed through a microfluidic chip with an embedded microfilter for further enrichment. With this method, about 99% of the WBCs were removed after the centrifugation process and high recovery rate of both DMS-79 (89%) and MCF-7 (99%) were achieved.

Although leading to more complex systems and processes with more challenges from the engineering perspective, a combination of multiple techniques can greatly increase the sensitivity and specificity of CTC detection and enable efficient CTC enrichment from clinical samples.

5 Conclusion

Blood-borne metastasis induced by CTCs is responsible for the majority of cancer-related deaths, making the detection and analysis of CTCs critical for developing effective tools for cancer diagnostics and therapies. While technologically challenging due to extreme scarcity of CTCs and the heterogeneity among them, enrichment of CTCs directly from blood samples of metastatic cancer patients can provide an alternative to invasive surgical biopsies and revolutionize personalized cancer therapies by serial “liquid” biopsies. Reliable CTC enrichment from clinical samples still is a technological challenge; however, significant advances were made in the past decade. In particular, multidisciplinary efforts that bring micro- and nanofabrication technologies to CTC enrichment introduced new approaches that improved both the sensitivity and the specificity of CTC detection over conventional laboratory procedures. Further advances in the field will require not only the development of new technologies to enrich CTCs but also rigorous testing of these technologies on patient samples and their clinical translation. Given the pace of technology development and intense research activity in the field, there

is every reason to be optimistic about the level of sensitivity and specificity that can be achieved with CTC enrichment tools and how these tools can be used to impact cancer research and patient care.

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Genetic Analysis of Circulating Tumour Cells

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1 Introduction

Great strides have been made in the classification and characterization of human cancers. Cancers are typically described based of their tissue of origin, histologic appearance and anatomical extent of disease, otherwise referred to as grade and stage, which yields valuable prognostic information. However, this system fails to fully explain the highly variable clinical behaviour of any one cancer, with significant heterogeneity seen among patients with the same cancer diagnosis (Fraser et al. 2015, Van't Veer et al. 2002). For instance, two newly diagnosed patients with metastatic castration-resistant prostate cancer (mCRPC) may be offered similar treatments and expected to have a similar prognosis, yet clinical experience dictates that these patients may have very different outcomes. Clinicians and researchers have been aware of this highly heterogeneous behaviour for years and have longed for other prognostic and/or predictive biomarkers. With the increased availability of advanced technologies, such as next-generation sequencing (NGS), that have been used to characterize the genetic events that lead to cancer, we are beginning to understand not only the molecular drivers of cancer, but also gain an appreciation for the extreme molecular heterogeneity underlying most cancers.

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Many cancers show different molecular subtypes that help to explain the varying clinical phenotypes observed (MacConaill and Garraway 2010; Biankin and Hudson 2011). Breast cancer is perhaps the earliest and best described example of this, with the expression of estrogen and progesterone receptors, and human epidermal growth factor receptor 2 (Her2) being used to stratify patients with early disease for their risk of relapse, and also as predictive markers for targeted therapies. More recently, a number of molecular subtypes of breast cancer have been identified, with gene expression assays clinically validated and being used as standard of care tests in some patients with early breast cancer to direct adjuvant therapy (Sørlie et al. 2001; Sotiriou and Pusztai 2009). Several other malignancies show similar molecular heterogeneity, with specific markers affecting either prognosis or response to therapy: *EGFR* mutations, and *ALK* and *ROS1* rearrangement in non-small cell lung cancer (Bergethon et al. 2012); microsatellite instability and *RAS* mutations in colorectal cancer (Guinney et al. 2015; Douillard et al. 2013; Le et al. 2015); *BRAF* mutations in melanoma (Chapman et al. 2011); and DNA repair defects in mCRPC (Mateo et al. 2015). Indeed, molecular heterogeneity appears to be the rule rather than the exception, with very few cancers showing homogenous molecular profiles (Biankin and Hudson 2011) (Fig. 1). Many investigators, including ourselves, envision a future that is driven by molecular diagnosis as opposed to the traditional morphologic diagnosis that is the current standard of practice. While such a vision, often referred to as "personalized" or "precision" medicine, is appealing, a number of barriers to this exist.

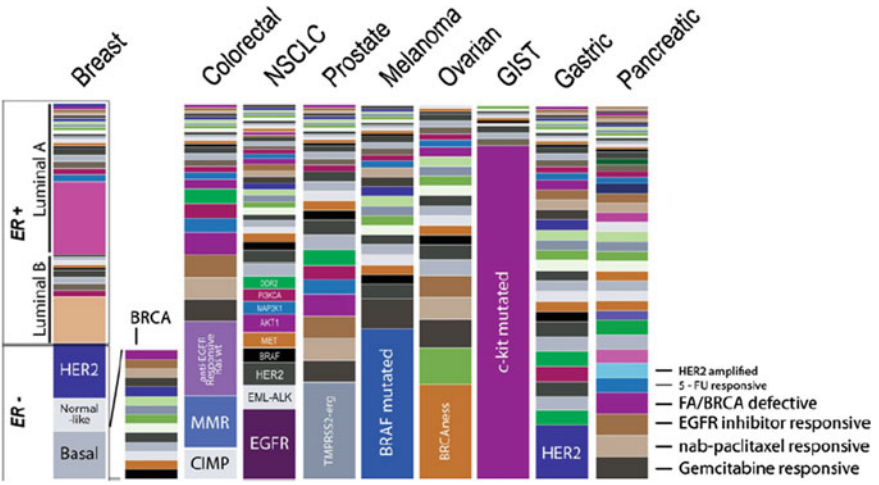

Fig. 1 Molecular stratification of human cancers: Most human cancers have many different genetic events leading to heterogenous molecular phenotypes, represented by each coloured striation. While some cancers have a common driver mutation, usually there is a so-called long tail of rarer mutations, often with varying prognosis and/or response to therapy (From Biankin and Hudson 2011, used with permission)

Acquiring suitable tissue to study is one such challenge. Fresh tissue biopsy is currently regarded as the gold standard for acquiring tissue for molecular characterization. In early stage disease, this is often readily available, as the vast majority of patients are diagnosed with cancer through fresh tissue biopsies and/or surgical resection, typically with excess tissue available. However, for patients with advanced metastatic disease, the use of tissue obtained at diagnosis may precede disease recurrence by several years and may not be representative of the current molecular phenotype of the malignancy, as clonal evolution may have occurred during the intervening period of time, and heterogeneity may exist between metastatic lesions and the primary tumour (Greaves 2012; Gerlinger et al. 2012). Yet many patients may not have metastatic lesions that are technically accessible for biopsy, and in those that do, biopsy may be associated with pain and anxiety, and is not free from risk. Furthermore, it would likely not be palatable for most patients to have multiple biopsies performed, both of different lesions and serially over time, as would be necessary to study inter- and intratumoural heterogeneity and clonal evolution.

With the identification of circulating tumour material, such as circulating tumour cells (CTCs), cell-free DNA (cfDNA) and tumour exosomes, there is hope that these may be used as blood-based surrogates for fresh tissue biopsy that is more palatable to patients and more readily available to researchers, and also provides a more representative sample from the overall tumour load. CTCs are particularly enticing, because these cells represent a key step in the metastatic process, and studying them may reveal crucial information about cancer metastases (Aceto et al. 2014). In this chapter, we will focus on the genetic analysis of CTCs.

2 CTC Enrichment, Isolation and Detection Technologies

An Australian physician, Thomas Ashworth, is credited with the first observation of CTCs; when performing an autopsy in 1869, he noted the presence of cells within the vasculature of a patient identical in appearance to cells within their cancer (Ashworth 1869). Since then, the vascular transit of malignant cells has been viewed as an essential component of the metastatic process (Fidler 2003). Epithelial cells arising from primary tumour tissue can gain access to the bloodstream by undergoing a series of morphological and molecular changes, in a process termed epithelial–mesenchymal transition (EMT). Through this, tumour cells display a range of phenotypes that allow resistance and dissemination to distant organs, thus playing a critical role in metastasis (Barrière et al. 2012; Denlinger et al. 2010).

CTCs are rare events and their biological features make isolation and detection a technical challenge, as they are outnumbered by white blood cells (WBC) in the order of 10^6–10^7 to one CTC per mL of blood. CTCs comprise diverse subpopulations that contribute to intratumoural heterogeneity and carcinoma invasion; therefore, a variety of different methods for their evaluation have been proposed over the years and yet a gold standard technique has been difficult to develop

(Alix-Panabieres and Pantel 2013, 2014). CTC enrichment can be achieved by the use of physical and biological properties leading to separation of tumour and hematopoietic cells. Their physical properties include cell size, density, deformability and dielectric characteristics, while biochemical properties refer to distinct cell-surface marker expression. Enrichment methods based on physical features are versatile, easy to implement at low cost and have the ability to isolate CTCs from any cancer type. However, the lack of specificity and unique features play a disadvantage as CTCs and WBC occasionally present with different sizes and shapes (Marrinucci et al. 2007). The ISET (Isolation by Size of Epithelial Tumour cells) system utilizes filters with 8-μm-diameter pores that allow the passage of small leucocytes but not larger carcinoma cells (Vona et al. 2000). Therefore, loss of small CTCs and collection of large WBCs present a limitation to this method. A commonly used approach consists of centrifugation of whole blood using density gradient media (e.g. Ficoll-Paque™, Lymphoprep™) generating an interphase layer of mononuclear blood cells (MNCs) and CTCs that can be easily collected for downstream analysis. Yet the high degree of contamination with leucocytes presents a difficulty by reducing CTCs purity to 1% or less. However, enrichment by depletion systems such as OncoQuick™ or SepMate™ in conjunction with RosetteSep™ may overcome these issues with greater recovery rates (Eifler et al. 2011).

Among their biological properties, CTCs can be enriched immunologically by expression of epithelial cell markers such as epithelial cell adhesion molecule (EpCAM) and cytokeratins (CK) (Armstrong et al. 2011). Additionally, the use of nuclear staining such as DAPI and leucocyte marker CD45 improves CTC detection by depletion. Therefore, the CellSearch™ system, a Food and Drug Administration (FDA)-approved method, represents a major achievement in the field by using EpCAM-coated ferromagnetic beads for isolation and immunocytochemistry for detection, achieving reproducibility and high performance (Riethdorf et al. 2007). Furthermore, the CTC-chip technology combines magnetic bead capture with microfluidic processing in a silicon chip that can isolate and confirm CTCs through staining and molecular characterization (Sequist et al. 2009; Nagrath et al. 2007). Other protein-based enrichment technologies include fluorescence-activated cell sorting (FACS) of cell surface and nuclear markers and immunomagnetic beads-based CTC adsorption such as EasySep™ for positive and negative selection (StemCell Technologies™).

Different methods for detection and quantification of CTCs have been established. Their advantages and limitations among the requirement of pre-enrichment steps lead to the use of combinatorial strategies (Krebs et al. 2014; Pantel and Alix-Panabieres 2012). Based on nucleic acid identification, the AdnaTest platform, a multiplex RT-PCR for a panel of genes, offers molecular characterization of CTCs in clinical diagnostics (Zieglschmid et al. 2005). Other detection assays include flow cytometry-based imaging and immunocytochemistry using multimarker identification (ImageStream®) (Lopez-Riquelme et al. 2013) and more recently, fluorescence imaging followed by isolation of cells by electric field changes within chip (DEP array®) (Gascoyne et al. 2009). Furthermore, the EPISPOT assay

provides an opportunity to distinguish between apoptotic and live CTCs; this is an in vitro functional assay based on immunofluorescence detection of secreted proteins as markers from viable cancer cells in short-term culture (Alix-Panabieres 2012).

Overall, the key challenge in CTC research stands by the scarcity and biological features of these rare cells for which a vast number of technologies worldwide are still in development. A potential solution to address the extreme rarity of the CTCs is the escalation of investigated blood volume, which is obviously technically challenging. One potential approach is the use of an anti-EpCAM antibody-coated metal wire, which is placed into a cubital vein for 30 min to catch CTCs out of the blood flowing past the wire (Saucedo-Zeni et al. 2012; Gorges et al. 2016). Initial results indicate that this strategy indeed increases CTC detection frequency of EpCAM-positive CTCs. Another interesting and less pre-selective option to analyse high blood volumes is diagnostic leukapheresis (DLA) (Fischer et al. 2013). By collecting peripheral mononuclear cells from litres of blood with continuous density centrifugation CTCs become co-collected and are enriched in DLA products. However, applying DLA in a routine clinical setting will require further research and optimization of the current approach (Stoecklein et al. 2016). Such studies are currently underway in a concerted fashion within the FP7 EU programme CTCTrap (http://www.cordis.europa.eu/result/rcn/150205_de.html).

3 Clinical Application of CTCs

Enumeration of CTCs with the CellSearch™ method has been cleared by the FDA for monitoring patients with metastatic breast cancer, colorectal cancer (CRC) and mCRPC. CTC counts before treatment initiation have been found to have prognostic significance and their changes during treatment to correlate with treatment outcome. A cut-off was determined for each of these tumour types (for breast cancer at ≥ 5 CTCs/7.5 mL, which was subsequently adopted and confirmed in mCRPC, and for CRC at ≥ 3 CTCs/7.5 mL) in order to differentiate patients with favourable and unfavourable outcomes. Interestingly, with minor differences, in all three tumour types patients can be separated into four groups based on the CTC changes during treatment as compared to baseline: (1) patients with favourable counts at baseline which remain favourable during treatment and have the best outcomes; (2) those with unfavourable pre-treatment counts who improve their counts during treatment to favourable levels and approach if not obtain the favourable outcome of the first group (3) those with favourable counts at baseline who deteriorate to unfavourable counts and obtain dismal outcome comparable to the last group; and (4) those who had unfavourable counts at baseline and maintain an unfavourable count during treatment who have the worst outcome.

3.1 Enumeration in Breast Cancer

CTC enumeration was first established as a prognostic tool in a study using metastatic breast cancer patients receiving chemotherapy. CTC measurements were obtained before and approximately 4 weeks after treatment initiation. CTCs were also measured in healthy women and those with benign breast disease, who served as normal controls. The study initially defined a boundary CTC count between favourable and dismal prognosis. For this purpose, thresholds of 1–1000 cells at baseline were systematically assessed for association with progression-free survival (PFS). This was found to differ already at 1 CTC/7.5 mL but reached a plateau at approximately 5 CTCs/7.5 mL. Additionally, none of the normal controls had ≥ 4 CTCs/7.5 mL rendering the limit of ≥ 5 as optimal choice for a prognostic marker.

Patients with ≥ 5 CTCs/7.5 mL were found to also have shorter median overall survival (OS) and PFS with multivariate analysis showing that CTC counts at baseline and at first follow-up after treatment were the most significant prognostic factor (Cristofanilli et al. 2004). Importantly, the level of CTCs at the first follow-up was also predictive of OS irrespective of the value at baseline, i.e., patients with a favourable CTC count at the first follow-up had also favourable median PFS and OS independently from an unfavourable baseline value. Vice versa, patients with unfavourable CTC count at first follow-up had unfavourable outcome independently of a favourable count at baseline.

3.2 Enumeration in Colorectal Cancer

In CRC, a disease with lower CTC yield than breast cancer, CTC counts were also systematically correlated with radiologic disease response in order to first determine an optimal time-point after treatment initiation for measuring CTCs, and second, to define an optimal cut-off to correlate with radiologic response (Cohen et al. 2008). This boundary was set at 3 CTCs/7.5 mL or less for a favourable prognosis, and the optimal time-point for measurement at 3–5 weeks after treatment initiation. Patients with unfavourable CTC counts at baseline, approximately 26% of the patients, had significantly shorter PFS and OS. The presence of an unfavourable count 3–5 weeks after treatment initiation was present in only 8% of the patients and was predictive of progression or death at the first disease evaluation 6–12 weeks after treatment initiation, albeit with a low sensitivity (27%) but high specificity (93%), indicating that while CTCs in CRC are able to identify a patient subset with a dismal prognosis, a large fraction of these poor prognosis patients are not detected. This may be due to low EpCAM expression on CTCs from these patients, which would not be detected by CellSearch^{TM} (Hardingham et al. 2015). Again, patients whose CTC counts converted from unfavourable to favourable achieved a similarly good PFS as those having favourable counts from baseline, with an improved OS, longer than those who did not convert to favourable counts, yet significantly shorter than those who maintained favourable counts from baseline. Importantly, radiologic response retained its predictive significance for OS within patients of the same CTC

prognostic group, as within a CTC group radiographic responders having a better outcome than non-responders.

3.3 Enumeration in Prostate Cancer

As opposed to CRC, in mCRPC high CTC counts are frequently observed. In the IMMC38 study [which lead to FDA clearing of CellSearch™ in this disease (De Bono et al. 2008)], patients initiating a new chemotherapy treatment had CTCs enumerated at baseline and at pre-specified time-points after treatment initiation. CTC counts were correlated with survival using as cut-off of ≥ 5 CTCs/7.5 mL for the unfavourable subgroup, which was adopted from previous breast cancer studies. An unfavourable count at baseline was again confirmed to be prognostic for a worse OS and patients who converted their CTC counts had a change in their prognosis consistent in the direction of their CTC count change. The prognostic significance of baseline and post-treatment CTC counts was independent of other established prognostic factors and the line of chemotherapy. Importantly, post-treatment CTC changes were found to be superior to PSA decrements of $\geq 30\%$ or $\geq 50\%$ in predicting OS. On a further study using the same data from the IMMC38 study, CTC counts were this time treated as a continuous as opposed to a dichotomous variable and even then were confirmed to be prognostic (Scher et al. 2009). A model incorporating baseline LDH and CTCs as well as post-treatment fold change of CTCs at 4, 8 or 12 weeks was reported as most predictive of survival and superior to PSA changes.

The prognostic significance of CTCs was also later confirmed with the data from the COU-AA-301 trial which tested abiraterone acetate versus placebo in mCRPC patients in the post-chemotherapy setting and had CTC counts incorporated (Scher et al. 2015). The combination of CTCs at 12 weeks post-treatment initiation and LDH at the same time-point was found to fulfil the Prentice criteria for surrogacy for OS. The surrogate composite marker categorized patients to low (CTCs < 5/7.5 mL, any LDH), intermediate (CTCs $\geq$ 5 CTCs/7.5 ml and LDH 250 U/L) and high (CTCs $\geq$ 5 CTCs/7.5 mL and LDH > 250 U/L) risk of death. This biomarker panel nevertheless needs to be validated in further prospective studies with other types of treatment before it becomes established as a surrogate marker for overall survival in clinical decision-making and as an endpoint for clinical trials.

3.4 CTC Counts as Clinical Decision Aid

The fact that early changes in CTCs after treatment initiation has prognostic significance instigated studies to test whether changing treatment early in patients who do not have a favourable CTC count after the first cycles of chemotherapy provides survival benefit. A study performed with this rationale in breast cancer (Smerage et al. 2014) failed to show any difference in OS between patients randomized to

early treatment change after unfavourable CTC count or continuation of the same treatment. This strategy remains to be tested in other tumour types.

3.5 CTC Counts in the Adjuvant Setting

CTCs can be detected in early non-metastatic cancers in the adjuvant setting in breast (Stathopoulou et al. 2002; Rack et al. 2014), colorectal (Sastre et al. 2008) and prostate cancer (Lowes et al. 2015), and seem to have prognostic significance. It is not clear, nevertheless, whether they provide additional information compared to the established prognostic variables to support decisions in this setting and their use has not yet been incorporated in the treatment of early cancer patients.

4 CTC Characterization

4.1 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Real-time RT-PCR is a highly sensitive method for detection and molecular characterization of CTCs. Danila et al. (2011) developed a sensitive PCR-based assay to detect *TMPRSS2-ERG* fusions in CTCs from mCRPC patients and explored the relationship between fusion detection and clinical outcome. Using TaqMan-specific probes, they detected *TMPRSS2-ERG* fusions in 37% of mCRPC patients consistent with previous reports. Though the presence of this gene fusion did not associate with clinical outcome, this study suggests that PCR-based analysis of CTCs can be used for clinically relevant biomarker research. In non-small cell lung cancer, a study by Maheswaran et al. (2008) was one of the first to show that *EGFR* mutations could be detected in CTCs. They used a microfluidic device to isolate CTCs, followed by Scorpion Amplification Refractory Mutation System (SARMS) technology and allele-specific PCR amplification to look at different *EGFR* mutations and compared the results with those obtained from concurrently isolated cfDNA from plasma and the original tumour-biopsy specimens. Their results showed that the CTCs analysis was more sensitive than cfDNA analysis, with the detection of CTC *EGFR* mutations in 92% of patients, while only 33% of patients had EGFR mutations detected in their plasma cfDNA. Serial CTC sampling also allowed them to detect the emergence of the *EGFR* T790M mutation known to confer resistance to EGFR-targeted therapies. A later study by the same group found that analysis of CTCs and cfDNA could be complimentary for the detection of *EGFR* T790M mutations (Sundaresan et al. 2016). Even though *EGFR* T790M genotyping was unsuccessful in 20–30% of cases, the combination of the two assays allowed them to identify this mutation in 35% of patients who had a negative or inconclusive tumour biopsies, suggesting that discordant genotypes between tissue and blood-based assays may result from technological differences as well as

sampling. Lately several groups have published the use of immunomagnetic AdnaTest to characterize CTCs by RT-PCR. Antonarakis et al. (2014) published an assay to detect androgen-receptor splice variant 7 messenger RNA (AR-V7) in CTCs from men with mCRPC and looked at the association with resistance to enzalutamide and abiraterone. They detected AR-V7 in CTCs of 39% and 19% of patients that had received enzalutamide and abiraterone, respectively, and showed that those patients had lower PSA response rates concluding that the detection of AR-V7 in CTCs from mCRPC patient could be associated with resistance to androgen-receptor-targeted therapies.

4.2 Fluorescence in Situ Hybridization (FISH)

Cytogenic studies based on FISH allow the study of specific gene amplification, deletion, copy-number variations and/or gene rearrangements in CTCs. Several studies have demonstrated the utility of this technique to identify certain biomarkers in CTCs. For example, in an early proof of principle study in 33 patients, Meng et al. (2004) showed that *HER2* gene amplification in breast cancer CTCs could be characterized by FISH, demonstrating 97% concordance between *HER2* status on CTCs compared to the primary tumour. Perhaps even more interesting, 9 of 24 patients who were initially identified as *HER2* normal at diagnosis acquired *HER2* gene amplification during disease progression, identified on FISH testing of CTCs. This was a critical finding as the vast majority of patients with metastatic breast cancer do not undergo biopsy to re-evaluate biomarker status. Importantly, four of these nine patients were treated with the *HER2* targeting therapy, trastuzumab, with three responding, providing further validation to the clinical relevance of this finding. Since then, several studies have used FISH to assess amplification, deletion, copy-number changes and/or rearrangements in CTCs from several tumour types including mCRPC. For example, Leversha et al. (2009) published the feasibility of using FISH to assess *AR* and *MYC* amplification in CTCs collected from 77 men with mCRPC using the CellSearch system™. They showed a high level of *AR* amplification in 37.5% of samples and a relative *MYC* gain in 55.8% in patients with 10 or more CTCs. Ten samples (13%) failed to give any FISH results due to high cell density, contaminating leucocytes or erythrocytes, poor cell morphology, cell loss during processing and/or poor FISH signal giving that specific study an overall success rate of 87%. Also, Swennenhuis et al. (2009) used 119 CTCs from 57 mCRPC patients to look at chromosomes 1, 7, 8 and 17 copy numbers. They observed extreme heterogeneity with respect to the aberrancy in the copy number of these chromosomes between patients, but also between CTCs from individual patients. In six of these patients, only diploid CTCs were identified; however, these patients all had a CTC counts of 1–5/7.5 mL using the CellSearch system™. This study also highlighted a key problem in the genetic analysis of CTCs: the majority of CTCs analysed (61%) did not provide FISH signals, with morphologic analysis of these cells suggesting they may be undergoing apoptosis.

CTCs can also be characterized by FISH for the presence or loss (heterozygous or homozygous) of phosphatase and tensin homolog (*PTEN*), assessed for the number of copies of the *AR* gene, or the presence of erythroblast transformation-specific–related gene (*ERG*)-based translocations. The simultaneous study of these genes by multicoloured fluorescence permits a comprehensive profiling of prostate cancer cells with prognostic value. Using FISH, Attard et al. (2009) showed that mCRPC CTCs, metastases, and primary tumour tissue had the same ERG gene status as treatment-naive tumours, suggesting this may be an early oncogenic event. They also observed homogenous *ERG* gene rearrangement status in CTCs in contrast to significant heterogeneity of *AR* copy-number gain and *PTEN* loss. A significant association between *ERG* rearrangements in treatment-naive tumours, mCRPC metastatic tumours, and CTCs and the magnitude of PSA declines in mCRPC patients treated with abiraterone acetate was also demonstrated. Using a new enrichment CTC platform (Epic Sciences) to evaluate *PTEN* gene status by FISH, Punoose et al. (2015) showed a concordance with the *PTEN* gene status assessed by immunohistochemistry in fresh and archival tissue in 62% of patient samples. CTC counts were prognostic, and *PTEN* loss in CTCs was associated with worse survival in metastatic CRPC samples.

4.3 Array Comparative Genomic Hybridization (aCGH)

To extend cytogenetic studies in individual CTCs, aCGH provides an established method to screen the whole genome for copy-number alterations (CNAs) (Fiegler et al. 2007; Fuhrmann et al. 2008; Möhlendick et al. 2013; Czyz et al. 2014). Modern oligonucleotide-based array CGH platforms can enable high-resolution CNA profiling of single cells, allowing a resolution of less than 100 kB in optimal single-cell experiments (Möhlendick et al. 2013). However, in contrast to FISH or certain targeted PCR approaches, CTCs need to be isolated and their genome amplified. Some basic principles and application examples will be outlined in the following section. A prerequisite to obtain whole-genome data from single CTCs is to isolate them from the background of contaminating normal nucleated blood cells that are present after every CTC enrichment method developed so far. The number of contaminating non-cancer cells is variable and unpredictable, but ranges usually between hundreds and several thousand cells (Stoecklein et al. 2016). Enriched CTCs can be isolated from contaminating normal cells in cell suspensions by three main approaches: (manual/automated) micromanipulation, fluorescence-activated cell sorting (FACS), and dielectrophoretic sorting (DEPArray™) (Stoecklein et al. 2016). Manual micromanipulation requires trained personnel, is laborious and can be limited by the subjectivity of the operator selecting an object of interest (Stoecklein et al. 2016). To overcome these limitations, (semi-)automated CTC isolation technologies have been developed. One solution is automated micromanipulation, e.g., with the AVISO CellCelector that has been applied for single CTC isolation captured with the MagSweeper device (Lohr et al. 2014) and in combination with the CellSearch™ system (Heidary et al. 2014). An alternative

automated micromanipulation system is the MMI CellEctor Plus that has been described for isolation of viable CTCs (Pizon et al. 2013). So far, no performance data (e.g. recovery of isolated cells) have been published for those (semi-automated) micromanipulation systems. Such data have been elaborated for FACS isolation of CTCs enriched with CellSearch™. Neves et al. (2014) reported a high correlation between CellSearch™ detection rates and FACS read-outs of the same cartridge. It was possible to recover 83% of the CTCs detected by CellSearch™ in 36 cartridges from metastatic breast cancer patients. In another study, Swennenhuis et al. (2013) reported a lower recovery rate of 41% in 10 lung cancer samples. The reason for the difference between the two reports remain unclear so far, but likely explanations include the use of different FACS instruments, different gate settings, or the morphologic and/or marker expression-differences on CTCs of breast and lung cancer, respectively (Stoecklein et al. 2016). The advantages of a FACS-based approach are the application of strict criteria (gating) to sort a defined target population and the speed of the isolation procedure allowing high-throughput studies. A disadvantage is the missing morphological control, which is provided by a technically very advanced dielectrophoretic isolation system, the DEPArray™. This technology was vigorously tested by Polzer et al. (2014) for isolation of breast cancer CTCs from CellSearch™ cartridges. Similar to FACS in breast cancer, the CTC recovery from the DEPArray™ was 77% (tested in 79 cartridges from 66 breast cancer patients) with excellent correlation between CTCs identified by CellSearch™ and the DEPArray™. The system is endued with advanced detection software, providing comprehensive documentation possibilities throughout the isolation process, and enables 100% specificity for sorted cells. Current disadvantages of the system are the unavoidable dead volume of the DEPArray™ cartridge of 29% leading to sample loss and a slower sample processing time compared to the faster FACS approach.

For the isolation of pure CTCs from filter-based enrichment, the aforementioned methods cannot be applied since captured cells tightly adhere to the filter membrane surfaces. Currently, the best isolation strategy appears to be laser-capture microdissection (El-Heliebi et al. 2013), which often requires manual inspection and can be quite laborious. A new development in filter technologies is the combination of self-seeding microwells combined with an automated "puncher" for cell isolation (Swennenhuis et al. 2015). The chips contain 6400 microwells with a single 5-μm pore in the bottom. Captured cells of interest are then isolated from the microwell by automatically punching out the filter bottom including the trapped cell. The overall single-cell recovery after filtering followed by punching was reported to be >70% using a breast cancer cell line. The microwell chips are currently designed to isolate pre-enriched rare cells, but development of similar chips to capture and isolate CTCs directly from blood is planned (Swennenhuis et al. 2015).

Because a single cell has only approximately 6 pg of DNA, genome-wide analyses of successfully isolated single CTC require whole-genome amplification (WGA). Currently, WGA is based on PCR, multiple displacement amplification (MDA) or a combination of both. PCR-based WGA comprises protocols using fully

or partially random primer sequences (primer extension pre-amplification (PEP)-PCR or degenerate oligonucleotide-primed (DOP)-PCR), and linker-mediated (LM)-PCR approaches, such as the GenomePlex kit based on random genome fragmentation and the deterministic Mse1 adapter-linker PCR (commercialized as Ampli1) during which the adapters are ligated to defined restriction sites. MDA is an isothermal amplification initiated by random priming DNA polymerase that has strand-displacement activity (usually phi29, alternatively Bst). Examples for combination methods are the PicoPLEX WGA kit and multiple annealing- and looping-based amplification cycles (MALBAC) method, which relies on displacement pre-amplification to generate PCR-amplifiable fragments (Macaulay and Voet 2014).

Although robust WGA protocols have been developed, it is important to realize that all available methods generate amplification artefacts to a certain extent. The expected types of errors are: (1) the selection bias mainly driven by local differences in CG content; (2) the drift bias due to random amplification events during the early cycles/stages of amplification resulting disproportioned amplicon representation in the WGA product; (3) complete allelic drop-out (ADO); (4) polymerase artefacts causing single-base errors; (5) generation of chimeric DNA sequences; (6) non-templated amplification; or (7) contamination (extensively reviewed in Sabina and Leamon 2015). These amplification errors can vary between the different WGA methods, and therefore, some methods are more compatible with certain downstream analysis, e.g. aCGH, than others (Macaulay and Voet 2014). Another important factor for WGA performance is the quality of the template DNA, which again requires a careful selection of the WGA method for the planned experiment (Sabina and Leamon 2015). For example, fragmented formalin-fixed DNA from clinical samples as CellSearch™ derived CTCs is less amenable for effective MDA, while good performance on such samples was achieved with LM-PCR.

However, several recent studies demonstrated that high-content genome analysis such as copy-number alteration (CNA) profiling using aCGH or low-pass NGS, whole-exome sequencing, as well as whole-genome sequencing can been successfully applied to WGA products generated from single CTCs. The two initial high-resolution CNA profiling studies of single CTCs used aCGH after the cells were isolated via micromanipulation from CellSearch™ cartridges of patients with metastatic CRC. While Heitzer et al. (2013) used the GenomePlex kit for WGA, Steinert et al. (2014) used the Mse1 LM-PCR. Additional mutational analyses on the WGA products complemented the aCGH analysis and provided evidence for the malignant nature of EpCAM+/CK+/CD45-/DAPI+CTCs captured by CellSearch™ in CRC. Generally, the CNA profiles displayed clonal relationships between different autologous CTCs as well as between CTCs and their matched primary tumours/metastases, but disclosed also genetic heterogeneity (Heirzer et al. 2013; Steinert et al. 2014). Two more recent studies established semi-automated workflows for CTC enrichment (CellSearch™) and single-cell isolation (FACS or DEPArray™) for comprehensive genomic profiling in the clinical setting (Neves et al. 2014; Polzer et al. 2014). Both studies used the deterministic Mse1-based LM-PCR (Ampli1) and showed that a complex series of genetic downstream

analysis with clinical relevance, including Sanger sequencing, genomic qPCR and aCGH could be applied to the very same WGA product. An important feature of these investigations was the implementation of quality control multiplex PCRs to check the genomic integrity after WGA before application of complex and expensive further genomic analyses. In summary, the studies demonstrated the malignant nature of CellSearch™ detected breast cancer CTCs and showed microheterogeneity between different CTCs isolated from the same patient. In addition, the work of Polzer et al. (2014) strikingly revealed that *ERBB2* is frequently amplified in CTCs of patients with HER2-negative primary tumours and that *PIK3CA* mutations mediating resistance against anti-HER2 therapies pre-exist in the same CTCs of these patients.

4.4 Next-Generation Sequencing (NGS)

As an alternative for aCGH, low-pass NGS-based CNA profiling can be applied to WGA products. This has been successfully performed on single and pooled CTCs isolated from CellSearch™ cartridges via DEPArray™ in small cell lung cancer patients (Hodgkinson et al. 2014). The advantage of such genome-wide NGS approaches is the possibility to precisely assess the key parameters of WGA performance, i.e. genome coverage, uniformity, reproducibility, unmappable read rates, chimera rates, allele dropout rates, false-positive rates for calling single-nucleotide variations, and the ability to call copy-number variations (Huang et al. 2015). In another study, Swennenhuis et al. (2013) performed whole-exome sequencing of MDA-generated WGA products of isolated single CTC from lung cancer patients and reported an average coverage at 20 × depth of 30% when sequencing to an average of 40 × depth with an overall amplification efficacy of only 25%. The disappointingly low NGS efficiency was most likely due to the poor performance of MDA on fixed cells from CellSearch™ cartridges and omitting to check genomic integrity as a marker for successful WGA. In contrast, Lohr et al. (2014) performed exome sequencing on unfixed (EDTA blood) MDA-amplified individual CTCs from prostate cancer patients and achieved better performance. Notably, low-pass (0.05 × coverage) whole-genome sequencing was used to assess the individual amplification bias and to predict which single-cell library would yield robust in-depth (>100 × coverage) exome sequencing data. Despite this, no single CTC exome was complete, and therefore, data from independent single CTC libraries were combined ("census" approach), which significantly reduced the false-positive rate of called somatic single-nucleotide variants. With this method, the authors identified common "trunk" mutations as well as private CTC mutations. Such non-overlapping mutations are of interest since they may provide novel insights into the evolution of tumour genomes or present novel targets for systemic therapies (Speicher and Pantel 2014). In contrast to MDA, but similar to Ampli1, MALBAC appears to be applicable to fixed CTCs captured by the CellSearch™ system: Ni et al. (2013) applied MALBAC for WGA on manually micropipetted single CTCs from lung cancer patients and observed characteristic

cancer-associated SNVs and indels in exome sequencing (mean target coverage at 20×: 45.5%, range 9.2–92.6) data from CTCs. These mutations provided information in the context of individualized therapy, such as drug resistance and phenotypic transition, but were heterogeneous from cell to cell. In contrast, CNA profiles obtained by low-pass sequencing (around 0.1 × sequencing depth), which were prepared from the same adaptor-ligated WGA product before the pooling step in exome library preparation, displayed a high degree of intra-individual homogeneity.

In summary, workflows have been established to comprehensively analyse the genomes of individual CTCs derived from clinical samples. But concerning exome or whole-genome NGS approaches, systematic benchmark testing of the different WGA methods is missing thus far. However, it is important to note that high-resolution profiling approaches have been successfully applied to CTCs isolated from the FDA cleared CellSearch™ system by independent groups and that potentially clinically relevant genetic information could be obtained from such samples. The direct analysis of cellular diversity and evolutionary trajectories in the CTC population might not only provide critical information for treatment decisions but will deliver relevant biological information on systemic disease progression in cancer.

5 Comparison of CTCs to Other Blood-Based Biopsy Methods

5.1 Circulating Cell-Free DNA (cfDNA)

Circulating cell-free DNA (cfDNA) is fragmented DNA (70–200 bp with larger fragments of about 21 kb), which is actively shed in the bloodstream from lysed apoptotic or necrotic primary tumour cells, metastatic cells, CTCs, normal stromal cells and nucleated blood cells (Fig. 2). Circulating tumour-derived DNA fraction in the sea of cfDNA varies between patients, ranging from 0.013% up to more than 90% (Bettegowda et al. 2014; Thierry et al. 2014). cfDNA has been shown to be a potential cancer biomarker in monitoring tumour progression and residual disease and also provides a useful liquid biopsy in patients that do not have detectable CTCs. Unlike capturing single CTC, which needs a combination of special equipment for cell enrichment and sophisticated single-cell sorter or cell picking technology, cfDNA extraction is cheaper, simpler and uses a standard plasma extraction assay. In recent years, many studies have focused on the utility of cfDNA on the molecular characterization of tumours using aCGH (Azad et al. 2015; Shaw et al. 2012), whole-genome sequencing (Mohan et al. 2014), exome sequencing (Jamal-Hanjani et al. 2015; Murtaza et al. 2013), targeted panel sequencing and epigenetic studies. Recent studies emphasize that the sensitivity and robustness of detecting copy-number aberrations depend on the fraction of the tumour-derived cfDNA and that the genomic aberrations have to be more common in the bulk of the

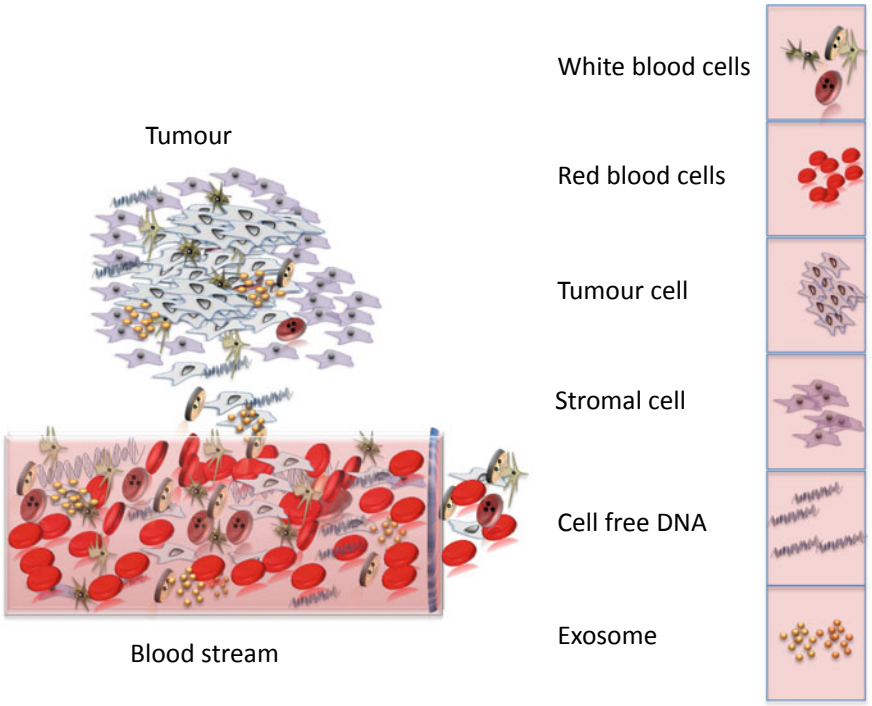

Fig. 2 Schematic of tumour derived products entering the bloodstream

tumour (Azad et al. 2015; Shaw et al. 2012). To improve sensitivity of cfDNA in detecting *EGFR* T790M mutation in non-small cell lung cancer fresh tissue, enrichment PCR-based approach combining cfDNA and CTC showed a better genotyping of *EGFR* T790M mutation across all the samples (Sundaresan et al. 2016). It has to be noted that the major drawbacks of the cfDNA approach towards personalized medicine and testing for drug susceptibility are the following: (1) the lack of defining details of tumour heterogeneity; (2) only relative copy number rather than absolute copy number can be obtained; (3) establishing in vivo and in vitro models such as cells line, organoids, animal xenograft modelling is not possible; and (4) lack of transcriptomic or proteomic assays.

5.2 Exosomes

Exosomes are small cell-derived vesicles (30–150 nm), which are present in all sources of body fluids, including urine, saliva, plasma, cerebral spinal fluid, breast milk and bronchial lavage fluid (Skog et al. 2008). They contain DNA, RNA, miRNA and protein similar to CTCs, and hence provide an alternative liquid biopsy to cfDNA and CTCs. Exosome-derived DNA is double-stranded DNA composed of

large fragments >2.5 kb, and exosome-derived RNA is possibly of better quality when compared to cell-free RNA (cfRNA) (Thakur et al. 2014). The field of exosome genomics and transcriptomics is in its early stages, but recently a report suggested that tumour-derived DNA is significantly higher when compared to cfDNA ranging from 56–82% and has a good coverage of 65–91% of the whole genome (San Lucas et al. 2016). Exosome extraction is not yet standardized, and the common use of ultra centrifugation may be beyond many diagnostic laboratories. However, similar to the cfDNA, exosomes lack the ability to establish in vivo and in vitro models.

6 Conclusions

Perhaps more than any other area of medicine, a push for "personalized" or "precision" medicine has defined cancer research in recent years. This has largely been due to the recognition of the highly heterogeneous nature of human cancers. Increased access to molecular characterization technologies including NGS has resulted in an unprecedented expansion in our understanding of the genetic events that drive cancer, with the ability to study cancers in ways that were previously not possible. Yet while the technology exists, obtaining suitable patient tumour tissue through biopsy is not always feasible, particularly when attempting to study clonal evolution or heterogeneity. Hence, an interest in the development of blood-based biopsy techniques could supplant fresh tissue biopsy.

The discovery of CTCs over a century ago was a landmark moment in understanding cancer metastases. More recently, these cells have been studied as a way to obtain tumour material, with a tremendous amount of research invested in CTCs and other circulating tumour material. While much has been learned about the significance of these biomarkers, much is yet to be discovered. CTCs enumeration provides valuable prognostic information and the molecular characterization and genetic analysis of CTCs appears to provide a valuable, non-invasive way to study cancer cells. Furthermore, the study of CTCs with other circulating tumour material may provide complimentary information. Whether these techniques will be accepted as a replacement to fresh tissue biopsies as a means of accessing tumour tissue remains to be seen, but there is reason for optimism. While significant barriers to this acceptance exist, blood-based biopsy techniques appear to be reliable and representative alternatives to fresh tissue biopsy.

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Quantitative Analysis of Circulating Tumor Cells Using RNA-Based Digital Scoring

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1 Introduction

The blood-borne metastasis of cancer is the leading cause of cancer-related deaths (Hanahan and Weinberg 2011). As such, CTCs provide a critical material for understanding the ability of tumor cells to intravasate into the bloodstream, either as single migratory cells or as clusters of tumor-derived fragments, survive in the circulation, and ultimately disseminate to distant organs and initiate proliferation (Poste and Fidler 1980; Allard et al. 2004; Yu et al. 2011; Stott et al. 2010; Aceto et al. 2014). While the vast majority of CTCs die in the bloodstream before giving rise to any metastatic lesion, the presence of these cells also provides a key opportunity to non-invasively sample cancer-derived materials during the evolution of the disease (Cristofanilli et al. 2004). The molecular composition of tumors evolves through the acquisition of genomic alterations and epigenetic modifications during disease progression and in response to therapeutic interventions (Easwaran et al. 2014; McGranahan and Swanton 2017). This may result in extensive tumor heterogeneity, a challenge facing the clinical management of cancer, and in the need to repeatedly adjust therapeutic regimens in response to acquired resistance

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mechanisms (Nardi et al. 2004). Much of our current information about cellular pathways involved in acquired drug resistance stems from repeat biopsies of tumors from patients on research protocols and from autopsy studies in patients who succumbed from extensive disease. However, the ability to repeatedly and non-invasively sample tumor cells through "liquid biopsies" may revolutionize the ability to tailor a patient's individualized therapy to address evolving tumor characteristics. Ultimately, blood-based tumor monitoring may allow early detection of invasive cancers and enable interventions before large tumor volumes may render curative treatment impossible.

Tumor-derived components in the blood are found as whole cells (i.e., CTCs), cellular fragments (exosomes or oncosomes), or free circulating tumor DNA (ctDNA). Each of these have unique properties relevant to their isolation and enrichment from whole blood, as well as the types of molecular information that may be derived from their analysis. ctDNA is comprised of nucleosome-sized DNA fragments that are primarily informative as to the genomic composition of tumors (Wan et al. 2017). Exosomes appear to contain a partial set of tumor-derived proteins, RNA, and even some DNA (Zhang et al. 2015). Both circulating DNA and exosomes are shed by both normal and tumor tissues, and cancer-derived molecules must therefore be distinguished, using molecular tools, from those shed by normal tissues. In contrast, whole tumor cells in the circulation are extremely rare, but their enrichment is dependent upon physical or cell surface marker expression (Nagrath et al. 2007). Once isolated, however, CTCs provide the full complement of the molecular information present within individual tumor cells. Across different cancer types, CTCs are estimated to range in number from 0 to 10 cancer cells per 10 mL of whole blood, amidst 10 billion red blood cells and 10 million white blood cells (Nagrath et al. 2007). CTCs are typically defined as cells that stain for epithelial cell-specific proteins (e.g., EpCAM and cytokeratins), with the exclusion of white blood cell markers (e.g., CD45) (Cristofanilli et al. 2009). More recent studies have used lineage-associated markers (e.g., PSA for prostate cancer), rather than epithelial markers that may be less specific for a given tumor type (Miyamoto et al. 2012). In addition, epithelial-to-mesenchymal transition (EMT), a cell fate switch associated with tumor invasiveness and drug resistance complicates reliance on epithelial markers to identify CTCs (Yu et al. 2013). We have therefore preferred CTC enrichment technologies that achieve "negative depletion" of blood specimens, essentially removing hematopoietic cells, while leaving CTCs untagged and unmanipulated in the product (Ozkumur et al. 2013). Microfluidic technologies currently achieve 10^4 to 10^5 enrichment of CTCs from whole blood specimens, resulting in a cancer cell population that may be 0.1–1% pure (depending on CTC abundance within an individual specimen). Given this success in rare cancer cell enrichment from blood, the challenge remains to score and molecularly characterize these partially purified cell populations. In this review, we focus on RNA-based approaches used for interrogating CTCs (Fig. 1) and their applicability in developing diagnostic assays that can be implemented in the clinic for monitoring therapeutic responses and ultimately for early detection of cancer in high-risk individuals.

2 RNA-In Situ Hybridization Identifies Epithelial and Mesenchymal CTC Populations

Immunofluorescence staining for tumor-specific protein markers are commonly used to study CTCs enriched from patient blood. A number of technical limitations have complicated such analysis, including the relatively low signal/noise ratio evident in CTCs within the context of contaminating blood cells, which often requires the combination of highly specific antibodies with secondary fluorescent antibodies for signal amplification, together with the rigorous setting of signaling threshold, image quantitation, and automated image scanning protocols (Allard et al. 2004; Alix-Panabières and Pantel 2014). Exemplifying this challenge is the high number of blood cells that simultaneously stain positive for epithelial as well as hematopoietic markers (cytokeratin/CD45 "dual positives"), which most frequently represent non-specific antibody binding, and which may outnumber true CTCs by multiple orders of magnitude (Stott et al. 2010). In this context, novel approaches to RNA-in situ hybridization (RNA-ISH) using multiplexed oligonucleotide probes present a powerful technology, with both a high degree of sequence specificity and quantitative amplifiable signal. For instance, several studies have used RNA-ISH against multiple probes marking epithelial and mesenchymal cell states to detect CTCs enriched using microfluidic isolation, filter-based methods or Ficoll gradients (Yu et al. 2013; Payne et al. 2012; Wu et al. 2015). These studies have shown that CTCs exist not only in uniquely epithelial versus mesenchymal states, but also in more complex conditions with simultaneous coexpression of both different numbers of epithelial and mesenchymal markers. Of particular interest, longitudinal analysis of blood samples from individual breast cancer patients receiving multiple courses of therapy may show dynamic shifts, with primarily epithelial CTCs as tumors respond to new therapeutic intervention, and the emergence of mesenchymal CTCs associated with acquired resistance and clinical therapeutic failure (Yu et al. 2013). The broad application of RNA-ISH technologies in characterizing individual CTCs in this way may provide a powerful tool for characterization of tumor cell heterogeneity during the course of cancer evolution and drug resistance.

3 RNA Sequencing Identifies CTC Subpopulations and Signaling Pathways Activated in CTCs

While RNA-in situ hybridization allows for more facile probe design than antibody-based detection, it is limited in throughput and in its ability to query only a few pre-selected transcripts of interest. RNA sequencing of either bulk-enriched or single-cell populations is technically challenging but enables interrogation of the entire transcriptome. Recent advances in next-generation sequencing technologies have made it feasible to sequence thousands of single cells from tumors, producing rich datasets that can more thoroughly probe the heterogeneity of cancer

(Tirosh et al. 2016). Whole transcriptomic analysis has been applied extensively to study primary and metastatic biopsies, but implementing this technology to CTCs has been complicated by the rarity of the cells and their condition after isolation using a variety of technologies (i.e., the need for unfixed cells with high-quality RNA).

The first approach to achieving CTC-specific transcriptional profiles used partially purified CTC populations subjected to single molecule (Helicos) RNA sequencing, subtracting RNA reads from matched control blood samples from that of the CTC-enriched cell populations (Yu et al. 2012). The Helicos single-molecular sequencing technology is unique in avoiding amplification of molecular templates, thereby providing highly linear measurement of transcript reads. Such subtractive strategies were best applied with CTC isolation platforms that capture cells on a fixed surface, from which single cells are not readily released but from which high-quality RNA can be isolated.

Indeed, early studies applying this strategy to pancreatic cancer CTCs in a mouse model, demonstrated increased non-canonical Wnt signaling in the CTC-enriched population (Yu et al. 2012), and an analysis of human melanoma CTC-enriched cell populations was noteworthy for cell motility-associated transcripts (Luo et al. 2014). However, deep sequencing of CTC-derived transcriptomes requires single-cell isolation and RNA seq, a strategy that has become possible with improving CTC enrichment technologies that allow for micromanipulation of CTCs that are unattached to a fixed surface.

For instance, the MagSweeper technology enabled single-cell RNA profiling of 87 cancer-associated genes in CTCs isolated from breast cancer patients, showing increased expression of the metastasis and EMT-associated genes (Powell et al. 2012). Using the microfluidic CTC-iChip technology, our own team has established

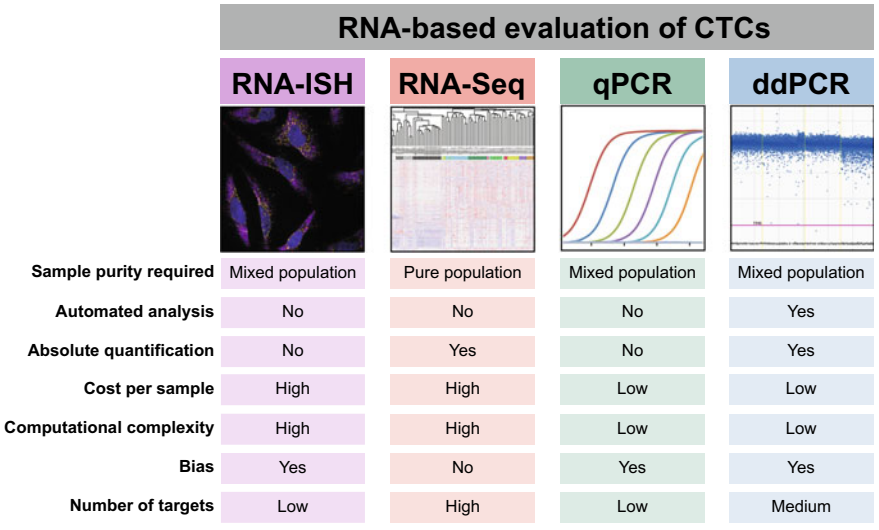

Fig. 1 Comparison of methods to study CTC-derived RNA

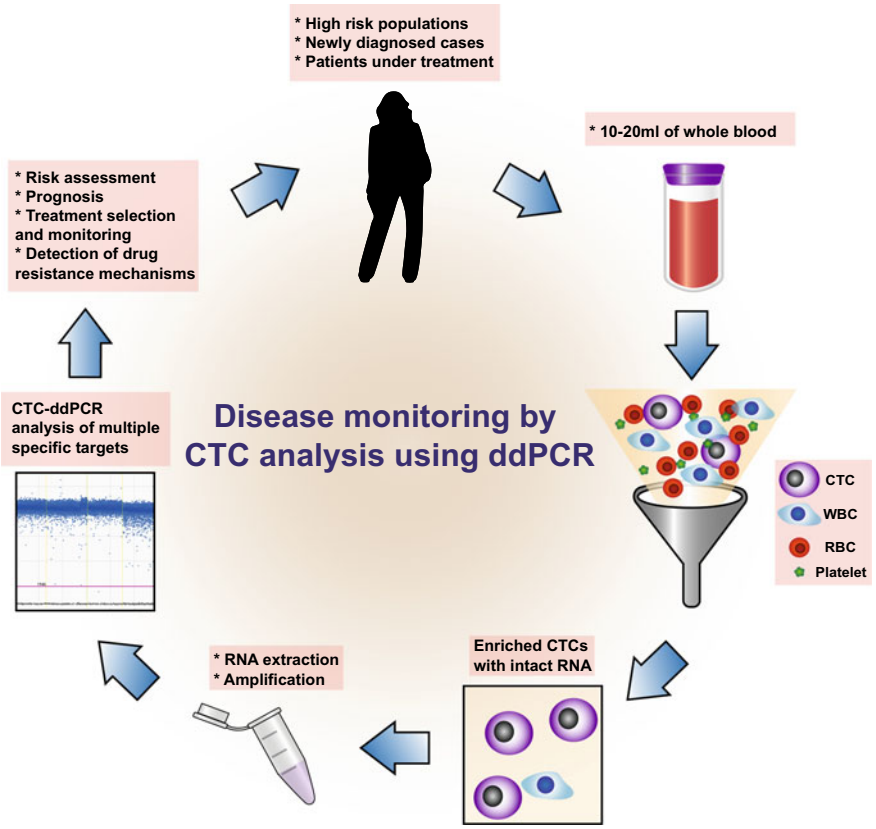

Fig. 2 CTC-ddPCR method

a platform through which hematopoietic cells are antibody-tagged and depleted, leaving behind unmanipulated CTCs with 10^4 to 10^5 enrichment (Ozkumur et al. 2013). The RNA quality in untagged, unfixed CTCs is very high, and the fact that cells are delivered in suspension facilitates micromanipulation of individual CTCs. This approach was used to define comprehensive transcriptomes of single CTC collected from both mouse models and clinical specimens. In a mouse model of pancreatic cancer, CTCs were highly enriched for expression of genes encoding the extracellular matrix proteins (ECM), compared with single cells isolated simultaneously from the primary pancreatic tumor (Ting et al. 2014). This aberrant expression of ECM proteins by cancer-derived cells in circulation is of particular interest in that it suggests the ability of metastatic intermediates/precursors to direct their own microenvironmental survival signals, which are characteristically provided by stromal cells within the primary tumor.

Single-cell RNA sequencing of CTCs traveling as individual cells versus those that are part of multi-cellular CTC clusters from the blood of women with metastatic breast cancer revealed >100 genes whose expression is relatively elevated in CTC clusters (Aceto et al. 2014). Among the top genes increased in expression within CTC clusters is Plakoglobin, encoding a protein belonging to the adherence junction complex. Plakoglobin is overexpressed >200-fold within CTC clusters, and its increased expression in primary tumors is associated with poor clinical outcome. Most importantly, in mouse models of breast cancer, knockdown of Plakoglobin does not affect cell proliferation, primary tumor formation, or release of single CTCs into the bloodstream; it does, however, profoundly suppress the generation of CTC clusters in the circulation and the generation of distant metastases in the lungs. Thus, Plakoglobin appears to be a key component of the cell junctions that helps tether CTC clusters together in the bloodstream, contributing to their enhanced metastatic initiation potential (Aceto et al. 2014).

RNA sequencing analysis of single CTCs from the blood of men with metastatic prostate cancer also identified mechanisms of resistance to therapies targeting androgen receptor signaling, including non-canonical Wnt signaling through Wnt 5A (Miyamoto et al. 2015). Moreover, these studies identified a profound level of heterogeneity in castrate-resistant prostate cancer, with distinct androgen receptor (AR) gene mutations and AR splicing variants present within different cells from the same patient. Indeed, single-cell CTC analysis is poised to reveal multiple independent mechanisms of acquired drug resistance, each of which may have different kinetics as patients receive successive lines of therapy.

Taken together, transcriptomic analysis of single CTCs may provide exceptional insight into the mechanisms driving tumor progression, metastasis, and acquired drug resistance. However, the effort and cost currently associated with single-cell RNA sequencing limits this platform to discovery and research applications. For widespread and routine clinical applications, more robust and economical RNA-based quantitative assays are also available and may present shifts in diagnostic paradigms for non-invasive cancer detection and monitoring.

4 High-Throughput Diagnostic Assays Using CTC-Derived RNA Signatures

RNA-based detection of CTCs within the background of hematopoietic cells relies upon the profound differences in transcriptional profiles between these cancer cells and surrounding leukocytes. Initial attempts at RNA-based detection of CTCs relied upon RT-PCR technology, with amplification of the prostate-specific PSA transcript applied to detect prostate cancer CTCs within the mononuclear cell fraction of blood samples, and the liver-specific albumin RNA similarly used to interrogate buffy coats from patients with advanced hepatocellular carcinoma (Kar and Carr 1995; Seiden et al. 1994). Additional markers, including mRNAs for cytokeratin, melanoma-specific markers, ALDH1, telomerase, MUC1, and others, have been

used to test for multiple additional cancer types (Ignatiadis et al. 2015; Gazzaniga et al. 2010; Arenberger et al. 2008; Shen et al. 2009; Pierga et al. 2007). Unfortunately, the success of these semi-quantitative RT-PCR-based analyses has been inconsistent in part because of their relatively low sensitivity and specificity in unpurified whole blood. A prevalence of 1 CTC per million leukocytes may be below the limit of detection using RT-PCR for a non-abundant transcript. Furthermore, even very low-level transcription by abundant hematopoietic cells of highly tissue-specific transcripts becomes a confounder when the tumor cells are present at such vanishingly low numbers. Indeed, higher numbers of contaminating WBCs increase the Ct values in qRT-PCR detection of identical amount of specific template, and large amount of non-specific template (equivalent of >1000 WBCs) produces SYBR Green noise independent of product amplification, interfering with the quantitative detection of the underlying signal (Pfitzner et al. 2014). The susceptibility of qRT-PCR to the inhibitory effects of large amounts of non-specific template may therefore explain the large variability and inconsistencies in reports describing CTC detection via this method. For all these reasons, we reasoned that initial enrichment of CTCs under RNA-preserving conditions, followed by quantitative digital PCR, provides a much more reliable strategy for RNA-based detection.

Droplet digital PCR (ddPCR) helps to overcome the inherent limitation posed by the presence of excess non-specific templates from contaminating cells by sequestering each individual cDNA template and PCR reagents into aqueous droplets within an oil suspension, thereby drastically increasing the effective concentration of the transcript of interest and allowing the differential expression of CTC-specific genes to be leveraged for identifying their presence (Fig. 2). Partitioning the entire cDNA sample into these droplets followed by high-cycle PCR to maximally amplify each template of interest creates a digital readout of the number of positive droplets, a measure of the prevalence of each transcript of interest (Kalinina et al. 1997). By tabulating the total number of positive and negative droplets and assuming the transcripts of interest follow a Poisson's distribution when partitioning into droplets, the absolute number of transcripts in the sample can be imputed. ddPCR has been successfully used for detecting rare alleles in the context of free plasma DNA, where its limit of detection is at allele frequency lower than 0.01% (Vogelstein and Kinzler 1999). In RNA detection, ddPCR may be somewhat less sensitive, but it robustly detects presence of aberrant splicing variants (e.g., the androgen receptor Arv7 transcript) in RNA purified from prostate cancer CTCs (Ma et al. 2016; Parkin et al. 2017). Beyond quantifying specific cancer-associated abnormalities, ddPCR detection also offers the potential for scoring and monitoring multiple normal lineage-specific transcripts that are absent from hematopoietic cells and hence denote the presence of CTCs from a given tissue of origin. Successful application of this strategy requires extensive validation of these transcripts to ensure complete absence of signal in normal blood cells, a feat that is greatly enhanced by the initial microfluidic enrichment of CTCs and reduced abundance of contaminating leukocytes.

The normal liver expresses unique transcripts, including albumin and multiple metabolic enzymes, that are completely absent from the expression profiles of other tissues, making it an ideal proof of principle for lineage RNA-based detection of CTCs. Targeting the detection of hepatocellular carcinoma (HCC), we recently established a panel of 10 RNA markers, optimized for ddPCR amplification from CTC-iChip-enriched whole blood of patients with known liver cancer. Total cellular RNA isolated from a 0.1–1% prevalent population of HCC CTCs amidst contaminating leukocytes was subjected to whole transcriptome amplification (WTA)—a step that exponentially increases the signal from all markers and also allows a limited amount of template RNA to be interrogated simultaneously for multiple markers, an important consideration given the known heterogeneity of cancer cells. Spiking individual HCC cells into whole blood followed by microfluidic enrichment and ddPCR showed the limit of detection to be 1 cell per 5 ml of blood, with millions of transcripts of interest generated from a single-spiked cancer cell (Kalinich et al. 2017).

Critical to the successful application of any diagnostic test is the comparison between positive cases and appropriate, age-matched, and risk-matched negative controls. As expected, our HCC digital CTC assay produced negligible background signals using blood samples obtained from young healthy donors. More importantly, it was similarly negative when applied to a cohort of patients with advanced chronic cirrhosis who were at high risk for the development of HCC and were on a regular screening protocol using serial measurements of the oncofetal antigen alpha fetoprotein (AFP) and ultrasound measurements. Among patients with confirmed HCC, the sensitivity of the assay was 56% at 95% specificity when tested against a cohort of patients with chronic liver disease at high risk for developing HCC. Using this assay, we were unable to detect signal without initial CTC enrichment, pointing to the importance of both debulking normal leukocytes and applying high-sensitivity digital PCR detection.

While these results constitute a proof of principle, they also open the door toward a viable CTC-based platform for monitoring and early detection of liver cancer. HCC arises predominantly in high-risk individuals with liver cirrhosis caused by infection with hepatitis B, hepatitis C, or non-alcoholic fatty liver disease (NASH). Currently, such individuals are monitored for plasma protein AFP levels, a sensitive but non-specific biomarker, which interestingly showed poor correlation with the levels of CTC-derived AFP mRNA. Tumor secretion of AFP protein and shedding of CTCs expressing AFP and other transcripts presumably measure different aspects of tumor biology, enhancing the likelihood that combining the two assays may increase predictive value for the early detection of HCC. Indeed, in an initial cohort of 15 patients with newly diagnosed HCC, 5 were positive for both AFP protein and CTC detection, 1 was only positive for AFP protein, and 4 were only positive by CTC assay. Thus, serial monitoring using both AFP and CTC quantitation should be tested as a novel blood-based screening platform for the early detection of liver cancer in high-risk populations.

RNA-based monitoring of CTCs has broad applications beyond the measurement of tumor burden in the blood. Judicious use of biomarkers can establish indices of intracellular signaling pathways, including androgen receptor (AR) signaling in prostate cancer or estrogen receptor (ER) responsive pathways in breast cancer. For instance, in women with metastatic hormone receptor-positive breast cancer, persistence of CTC-derived transcripts indicating ER signaling despite treatment with ER-targeting therapy identifies patients likely to have rapid progression on endocrine therapy (Kwan et al. 2018). Such digital quantitation of CTC-derived RNA provides the first non-invasive blood-based pharmacodynamic measurement of ER signaling following breast cancer therapy. In addition to studies of metastatic breast cancer, in a cohort of women with early stage breast cancer, elevated CTC-derived RNA signal after initial courses of presurgical (neoadjuvant) chemotherapy was predictive of the presence of minimally residual disease at the time of surgical resection (Kwan et al. 2018). In analogous studies of prostate cancer, CTC-derived signal for the AR splicing variant AR-V7 and for the HOXB13 biomarker in men at first relapse of metastatic prostate cancer were highly correlated with abbreviated clinical response to the androgen synthesis inhibitor abiraterone. In men with localized prostate cancer, detectable CTC-derived RNA signal is correlated with extracapsular (seminal vesicle) invasion and metastasis to regional lymph nodes (Miyamoto et al. 2018). Digital quantitation of CTC-derived transcripts is also applicable in melanoma, where neural crest and carcinoembryonic antigen-associated RNAs provide robust signal of circulating cancer cells. In metastatic melanoma, serial monitoring of patients receiving immune checkpoint blockade shows a highly significant correlation between early declines in digitally quantified CTC burden and subsequent response to immunotherapy and overall survival (Hong et al. 2018). Finally, across many different types of cancer, oncogenic translocation products leading to chimeric transcripts are detectable using CTC-derived RNA analysis, leading to the appropriate application of targeted therapeutic regimens. Taken together, the convergence of high-quality enrichment of CTCs with intact RNA together with high-sensitivity RNA-based digital PCR provides new tools for the effective monitoring of cancer cells in the blood.

5 Concluding Comments

Liquid biopsies, defined as the interrogation of blood components to ascertain the properties of solid tumors, are poised to revolutionize the diagnosis and treatment of cancer. Among the multiple technologies, from circulating plasma DNA to exosomes and CTCs, each has its unique strengths and weaknesses, and each may play a greater or lesser role in a specific clinical scenario relevant to a particular tumor type. In general, ctDNA has had the benefit of ease of collection and analysis, but has been limited by the analysis of genetic variations in tumors; in contrast, CTC analyses have been limited by the technological hurdles in rare cell isolation and the biological features involved in their molecular characterization. We believe that

new approaches involving automatable microfluidic negative depletion of normal blood cells to enrich for untagged and unbiased CTCs, together with RNA-based digital readouts are now poised to level the playing field, brining CTC measurements along with ctDNA into the frontline of clinical applications. These two types of liquid biopsies are highly complementary, as illustrated by the hypothetical scenario of a mutation of unknown origin identified using ctDNA, whose organ of origin may be identified by RNA-based CTC analysis. Moreover, some cancers are driven by defined genetic alterations readily identified by ctDNA, while others may be tied to epigenetic features or transcriptional changes that are invisible to DNA sequencing, but apparent by RNA-based analysis. Thus, we envision a future in which liquid biopsies with distinct capabilities may be integrated to provide a comprehensive non-invasive platform for monitoring cancer, ranging from the earliest evidence of cancer initiation or recurrence, to guiding the most effective therapeutic options for evolving cancer resistance. Finally, measuring transcriptional programs as the direct output of the genetic and epigenetic drivers promises to improve our ability to understand tumor biology and respond to its changes, opening the doors to more effective ways to diagnose, treat, and monitor cancers in the future.

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Circulating Tumor Cells: High-Throughput Imaging of CTCs and Bioinformatic Analysis

Kevin Keomanee-Dizon, Stephanie N. Shishido and Peter Kuhn

1 Introduction

In 1869, Ashworth presented a preliminary analysis of circulating tumor cells (CTCs) and its implications for understanding cancer metastasis (Ashworth 1869). With few exceptions, this observation was ignored until the late twentieth century, in large part because CTCs act at ultra-low concentrations in the bloodstream: in the range of 1 in 10^9 blood cells. Recent technological developments, however, have now made it possible to identify CTCs from noninvasive liquid biopsies, and while the field is still much in its infancy, CTC frequency has already been prognostically linked to overall survival in metastatic breast, colorectal, and prostate cancer (Budd et al. 2006; Cohen et al. 2008; de Bono et al. 2008). But even if we can detect CTCs, *any* effort in using them to study cancer progression dynamics has a number of other obstacles. One worth discussion is tumor heterogeneity: the emergence of different cellular phenotypes and enigmatic cellular interactions inflicted in the tumor microenvironment and circulation—posing significant challenges for treatment decision-making. Although there have been waves of CTC detection tech-

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nologies, each with its own successes, most rely on protein enrichment or physical selection methods (Ozkumur et al. 2013; Yap et al. 2014). Since there is no general CTC biomarker consensus (Phillips et al. 2014; Samson and Baas 2015), such size-specific or protein-based assumptions are dangerous, as potentially relevant cellular events could inadvertently be lost through these conventional assays. Thus, the search for *which* tumor cells are critical for disease progression may be missing something essential.

In stark contrast to most CTC detection methods, the high-definition single-cell assay (HD-SCA) workflow was designed as an enrichment-free, high-throughput assay for the *entire* population of cells in a liquid sample, while at the same time being fully compatible with clinical pathology. Founded by the Scripps Physical Sciences-Oncology Center (PS-OC[1]), the HD-SCA workflow brings together modern methods of immunofluorescence with more sophisticated image processing to rapidly and accurately detect rare tumor cells among the milieu of platelets, erythrocytes, and leukocytes in the peripheral blood. This approach has been established as a reliable and sensitive CTC detection and characterization workflow for metastatic breast, lung, and prostate cancers (Marrinucci et al. 2009, 2012; Nieva et al. 2012; Pecot et al. 2011), with direct clinical applications (Carlsson et al. 2014; Dago et al. 2014; Gross et al. 2015).

The purpose of this chapter is to explain how the HD-SCA technology endows visualization, characterization, and measurement of rare CTCs from liquid biopsies, and what the resulting information teaches us about how cancer spreads through the human body. We introduce the basic concepts of the HD-SCA workflow, focusing on topics relevant to image processing and analysis; show how this technology has been used to investigate and measure the liquid phase of cancer metastasis; and explore how the HD-SCA technology, together with the current standard of care, has implications for improving the precision of cancer diagnostics and interventions.

2 Standardized Blood Cell Preparation and Immunofluorescent Staining

Advancing our knowledge of the spatiotemporal evolution of cancer in the human body requires direct quantitative access to CTCs—potential "seeds" responsible for the metastatic cascade (Chaffer and Weinberg 2011; Scott et al. 2012). Ideally, we would like to measure all the heterogeneous CTCs and their evolution, both as the disease naturally progresses and under treatment pressure. As such, each blood biopsy is treated with erythrocyte lysis and then, with much care, *all* nucleated

[1]To promote a physical sciences perspective of cancer, the US National Cancer Institute's PS-OC Program was launched in 2009—initially a Network of 12 Centers, including the Scripps PS-OC—with the aim of converging traditional cancer biology and oncology with the physical and engineering sciences to bring radical new approaches to cancer research (http://physics.cancer.gov).

blood cells are plated as a biological monolayer onto custom glass slides [as published in Marrinucci et al. (2012)]. The glass slides have a proprietary adhesive coating that enables maximum retention of live cells, holding $\sim 3 \times 10^6$ nucleated cells per slide. After plating, all cells are fixed, permeabilized, and immunofluorescently stained with monoclonal antibodies targeting a panel of cytokeratins (1, 4, 5, 6, 8, 10, 13, 18 and 19), an intermediate filament found exclusively in epithelial cells; CD45, a pan-leukocyte-specific marker; 4′,6-diaminido-2-phenylindole (DAPI), a nuclear stain; and—if desired—an additional preselected, disease-specific fourth marker (such as androgen receptor (AR), estrogen receptor, HER2, PDGFRα, VE-cadherin). Finally, each slide is subsequently imaged via a custom fully automated scanning microscopy system to provide a snapshot of the dynamical disease (see Fig. 1 for an overview).

Although this approach requires chemically fixed cells, a process with many known limitations (Phillips et al. 2014), it has the advantage of requiring no enrichment step and minimal processing of blood samples. The method can thus be applied to characterize the entire heterogeneous circulating cell population from

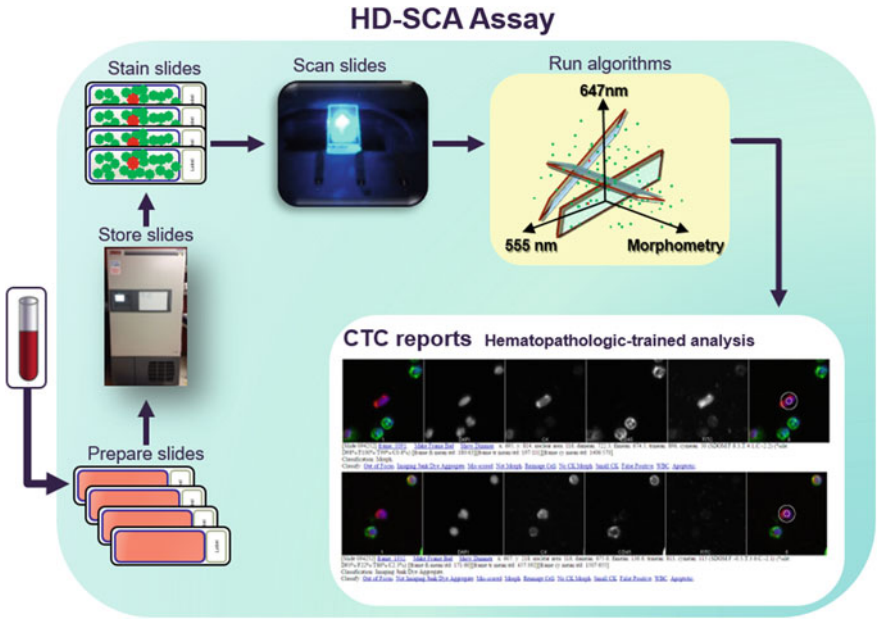

Fig. 1 A schematic overview of the HD-SCA workflow. Received patient whole blood is treated with erythrocyte lysis and then plated onto adherent slides. Multiple slides (from the same patient draw) are kept and preserved in a biorepository until analysis is desired. This provides researchers with the ability to assay the same sample using several strategies at any time. When slides are ready to be assayed, they are immunofluorescently stained and imaged via automated scanning microscopy. The resulting images are computationally analyzed to infer candidate CTCs, which are then presented in reports for classification by a hematopathology-trained specialist

low volume samples without any selection bias, efficiently and inexpensively. Additionally extensive pre-analytical variable testing has been conducted to ensure accurate and reproducible data output (Rodriguez-Lee 2018; Stephanie accepted)

3 High-Throughput Imaging of CTCs

The advent of fast and reliable automated fluorescence microscopy has granted us measurement of multiple quantitative descriptors over large populations of cells in the peripheral blood, down to the single-cell level (Marrinucci et al. 2012; Nieva et al. 2012; Cho et al. 2012; Kuhn and Bethel 2012; Wendel et al. 2012). In concert with the immunofluorescent staining protocol described above, a custom-designed optical microscope and imaging pipeline make it possible to analyze up to 3×10^5 cells per second. In the following sections, we outline the high-throughput imaging aspects of the HD-SCA workflow, starting with the scanning microscopy system and proceeding through a succession of computational steps on the path to the high-definition single-cell assay (Fig. 2).

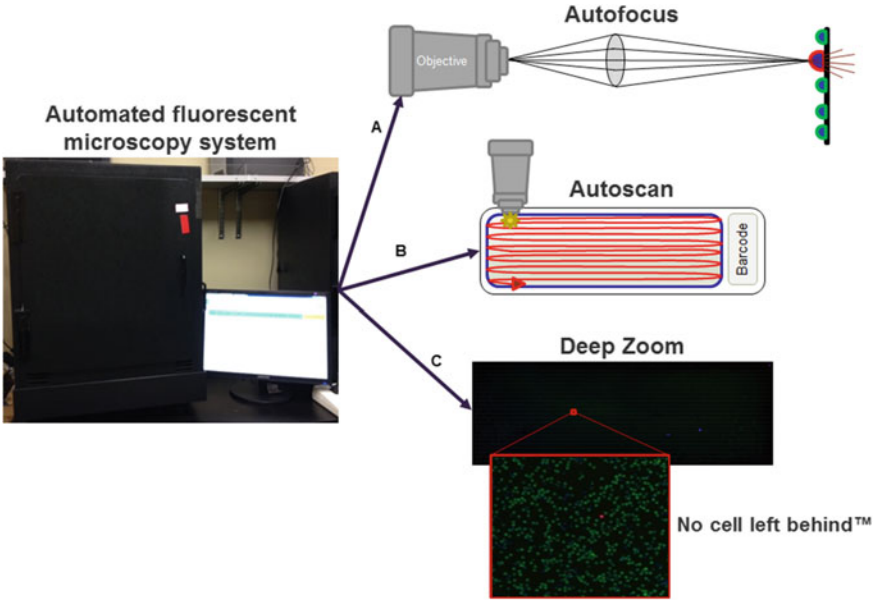

Fig. 2 HD-SCA automated fluorescence scanning microscopy system. **A** First, the focus and exposure (for each fluorescent channel) are automatically set for each slide. **B** Second, the entire active surface of each slide is automatically scanned, and then, each cell is automatically segmented for extraction of cellular features. **C** Finally, a deep zoom is created from the collection of scanned images of the slide, allowing researchers to interactively pan around and zoom in/out

4 Automated Fluorescence Imaging

The custom fully automated scanning microscopy system is equipped with a broad-spectrum illuminator; a multiband filter set (optimized for DAPI, fluorescein isothiocyanate (FITC), tetramethylrhodamine-5-isothiocyanate (TRITC), and Cy5, as well as other similar fluorophores); and a 20 MHz, 14-bit camera with an IEEE 1394 interface for high-speed information transmission. Its partner in crime, a homemade imaging analysis pipeline, is an equally important aspect for automatically acquiring and analyzing digital images. Housed with mass computing storage and processing power, this optical setup is poised for high-throughput fluorescence imaging.

A fundamental first step for analysis of cellular microscopy-based assays is focusing of nuclei. Accordingly, the HD-SCA imaging pipeline uses an autofocus algorithm based on Vollath's autocorrelation function (Vollath 1987), which, based on the comparison of 13 focus algorithms for fluorescence microscopy, has been shown to be an optimal (Santos et al. 1997). Second, autoexposure is performed to automatically set the exposure time to within an ideal range for each optical channel (this is in parallel a method for normalizing experimental variance between slides). Altogether, a full scan at 10× magnification of the entire active area of a slide—that is, 19.3 × 56.9 mm and $\sim 3 \times 10^6$ data points—takes 45 min.

The imaging system's stage is engineered to support 4 slides, ultimately producing over 6900 digital images of upward to 10^7 cells (Marrinucci et al. 2012). Acquired raw images are then supplied to arrays of hard drives (RAIDs) that have built-in redundancy. The resulting images, which retain fine cellular details of nuclear and cytoplasmic structure, are then fed into a two-part semi-automated algorithm to identify candidate CTCs.

5 Automated Measurement and Detection of CTCs

There are many well-established image processing programs for cell segmentation and feature extraction of microscopy-based cellular assays (for instance, CellProfiler and ImageJ, as well as programming environments/languages such as LabVIEW, MATLAB, Python, and R). The open-source nature and generality of ImageJ have made it an attractive image processing and analysis framework for the HD-SCA workflow. The combination of ImageJ and more advanced algorithms (via Python) enables automated analysis of phenotypic features from thousands of images.

Starting with single-cell segmentation to locate cells and boundaries in images, the algorithm identifies each cell from digital imagery by the DAPI intensity of a given nucleus. Each cell's center of mass is then computed to generate masks for quantitative descriptors of cellular phenotypes (Marrinucci et al. 2012), including physical characteristics (area, aspect ratio, circularity, roundness, solidity, cluster count) and expression levels (fluorescent signal intensity) for every nucleated cell,

in each optical channel (fluorescence excitation wavelength (λ_{ex}) = 359 nm and fluorescence emission wavelength (λ_{em}) = 461 nm for the DAPI fluorophore; λ_{ex} = 555 nm and λ_{em} = 578 nm for the TRITC (cytokeratins) fluorophore; λ_{ex} = 647 nm and λ_{em} = 666 nm for the Cy5 (CD45) fluorophore; λ_{ex} = 495 nm and λ_{em} = 519 nm for the FITC (optional, disease-specific) fluorophore) (Table 1). If further analysis for a cell of interest is desired, the cell's recorded center of mass, or equivalently, its coordinates on the slide, can easily be mined and thus sequestered on any calibrated system for downstream characterization. As an example, cells of interest can be reimaged at different focal planes with a confocal microscope to obtain 3D information (see Fig. 4; for more examples, see HD-SCA Beyond Fluorescence Section below).

Like all cellular assays, the usual caveats apply: Fixation, permeabilization, and staining will inevitably vary. But all of these variables are certainly tractable and manageable by normalizing each rare (CTC) measure with the surrounding nonrare (leukocyte) measures to generate relative CTC metrics within the same experiment. These relative metrics are then computationally analyzed on a cell-by-cell basis, primarily via the fluorescent signal intensity of cytokeratin and CD45, to infer candidate CTCs, which are then passed to a specialist for technical analysis and classification.

Table 1 CTC measurements automatically generated with the HD-SCA workflow

Feature	Description	Units
Physical characteristics	Physical measurements generated for each nuclei	
x	Center of mass *x* (*x*-axis)	16-bit pixel value
y	Center of mass *y* (*y*-axis)	16-bit pixel value
Roundness	How closely the shape of an object approaches that of a circle	
Circularity	Inverse of roundness	
Area	Area size	16-bit pixel value^2
Area (local ratio)	Ratio of the area of nuclei of interest to the average area of the surrounding 50 nuclei	
Aspect ratio	Relationship between width and height	
Solidity	Texture	
Cluster count	Individual cell nuclei within a CTC aggregate	Cell nuclei
Expression-level measurements	Signal intensities generated for each cell, in each fluorescent channel (DAPI, TRITC, FITC, Cy5)	
Mean	Mean pixel intensity of the cell of interest	16-bit pixel value
Standard deviation	Standard deviation of the mean pixel intensities of the cell of interest and the surrounding cells	16-bit pixel value
Standard deviation over the mean	The number of standard deviations over the mean pixel intensity for the cell of interest to the mean pixel intensity of the surrounding 50 cells	16-bit pixel value

6 No Cell Left Behind™

Cancer evolves in the patient from initiation to widespread metastatic disease through a series of subclinical processes that span a wide range of temporal and spatial scales. Broadly speaking, cancer must act in ways that are coherent in the context of Darwinian dynamics and in relation to the biophysical events in the patient's body. Cancer cells must sense, compute, and make decisions: to find food, to travel through the bloodstream, to evade our immune system, to act collectively (or compete) with other cells, and to proliferate—behavior must be "functional." In theory, there are many ways in which such functional information could be represented (and thus measured) during the development of cancer metastasis. One possibility is that CTCs represent metastatic seeds with varying degrees of malignancy (Chaffer and Weinberg 2011; Scott et al. 2012). But how much information does a CTC tell us about the disease? What biological signals are important? Answering these questions is crucial because, while there is a general consensus that CTCs express epithelial markers, less is known about cellular states associated with CTCs expressing low epithelial levels, small CTCs, the life span of CTCs, CTC aggregates, and the implications of such phenotypes on disease evolution. These ideas point to the need for detection and characterization of the largest diversity of candidate CTC populations across a wide range of clinical stages, which is made possible with the HD-SCA workflow. This approach has worked exceedingly well for quantifying several morphological and cellular properties of the circulatory phase (Marrinucci et al. 2009, 2012; Nieva et al. 2012; Carlsson et al. 2014; Dago et al. 2014; Gross et al. 2015; Kuhn and Bethel 2012; Wendel et al. 2012; King et al. 2015; Phillips et al. 2012a, b; Ruiz et al. 2015; Marrinucci et al. 2007, 2010; Lazar et al. 2012; Chalfin et al. 2018; Malihi et al. 2018; Williamson et al. 2016; Carlsson et al. 2017; Gross et al. 2015), and technical analysis by a hematopathology-trained specialist has been central to this success. In this section, we turn our focus to the high-definition single-cell assay and classification of candidate CTCs. Throughout, we emphasize a fundamental understanding of CTCs in human cancers is critical for using them as biomarkers.

6.1 High-Definition Single-Cell Assay and Classification

In the second part of the semi-automated algorithm, candidate CTCs are further analyzed by a hematopathology-trained specialist. For contextual comparison, digital images of candidate CTCs are presented with surrounding nonrare cells within the field of view in each optical channel—and overlaid for reference—examples of which are presented in Fig. 3. For quantitative comparison, relative metrics of physical characteristics and expression levels of candidate CTCs are also shown, again for each optical channel. Multiple phase spaces, such as morphometry, cytokeratin intensity, and CD45 intensity, can thus be analyzed simultaneously at the single-cell level. At present, candidate CTCs are classified into four broad

Table 2 CTC classification schema

Type	Description
HD-CTC	Putative HD-CTCs are defined by having an intact nucleus, identified through DAPI; being of epithelial origin, characterized by a bright cytokeratin (CK) stain; no CD45 signal—a leukocyte marker; and a distinct morphology from the surrounding leukocytes. HD-CTCs must have a clear circumferential cytoplasm containing the entire nucleus. See Panel A in Fig. 3
CTC-small	CTC-small expresses CK at appropriate levels to be a HD-CTC, but has a similar (or smaller) nuclear size in relation to surrounding leukocytes. See Panel D in Fig. 3
CTC-low CK	CTC-low CK expresses an insufficient level of CK to consider a HD-CTC, but has a significantly larger nucleus compared to surrounding leukocytes. This group is potentially associated with cancer stem cells or cells undergoing an epithelial-to-mesenchymal transition. See Panel E in Fig. 3
CTC-cfDNA producing	Candidates with identifiable apoptotic changes, such as cytoplasmic blebbing and/or nuclear fragmentation. See Panel F in Fig. 3

categories; see Table 2 for detailed description[2] and Fig. 3 for high-definition visualization of each category.

7 Morphometry of CTCs

Cellular morphology has been used historically to discern the malignancy of a cell (Tosi et al. 1986). Abnormalities in nuclear size, microstructure (including chromatin organization and mitotic figures), and shape are morphologic hallmarks associated with cancer pathology (Partin et al. 1992; Pienta and Coffey 1991). In the context of the fluid phase of solid tumors, a breakthrough case study of the morphometry of CTCs in a patient with well-differentiated lung adenocarcinoma was found to be strikingly similar to the epithelial cells of the primary tumor: CTCs were larger in size relative to leukocytes and exhibited low nuclear-to-cytoplasmic ratios (Marrinucci et al. 2009). In a larger study of breast and colorectal cancer patients, CTCs shared heterogeneity consistent with cancer cells from other spatial regions (such as the primary and metastatic tumor sites) in the body, including high and low nuclear-to-cytoplasmic ratios, as well as early and late apoptotic changes (Marrinucci et al. 2007, 2010).

While it is necessary to study cancer biology in model systems, it is enormously challenging to address the issues that arise in realistic contexts, e.g., the complexities of fully natural signals in the human with cancer and the dynamic biological processes upon which the disease is acting. To support the cell biology

[2]Some care is needed here, and it is worth noting that CTC classification is under ongoing development and rigorous investigation. Thus, Table 2 is by no means a complete list (!).

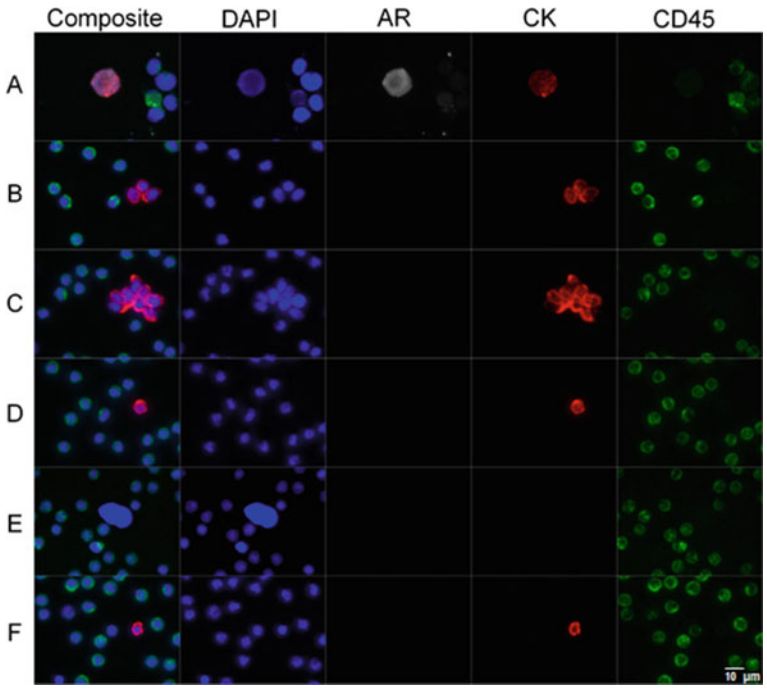

Fig. 3 Gallery of representative CTCs detected in the blood of a patient with prostate cancer. Panel **A** shows a composite image and individual optical channels of an HD-CTC. In addition, this particular tumor cell expresses androgen receptor (AR). HD-CTCs are defined by having an intact nucleus, identified through DAPI (blue); being of epithelial origin, characterized by a bright cytokeratin (red) stain; no CD45 signal (green)—a leukocyte marker; and a distinct morphology. Panel **B** represents a CTM triplet. Panel **C** represents a mega-CTM (>5 CTCs). Panel **D** represents a CTC-small. Panel **E** represents CTC-low CK. Panel **F** represents CTC-cfDNA producing. Scale bar = 10 µm

community in translating such studies, the HD-SCA assay was used to morphologically compare CTCs from patients with prostate cancer and prostate tumor cells derived from an LNCaP cell line (Lazar et al. 2012). Lazar et al. demonstrated important differences between the average total cell areas of actual patient CTCs ($\sim 90 \pm 50\ \mu m^2$) and LNCaP cells ($\sim 140 \pm 50\ \mu m^2$)—as well as differences in the expression levels of cytokeratin and AR—providing translational benchmarks for experiments in classical model systems.

More recently, the HD-SCA workflow was applied to a cohort of metastatic melanoma patients, where circulating melanoma cells (CMCs) were discovered to be, on average, 1.5-fold larger than surrounding nonrare nucleated blood cells (Ruiz et al. 2015), an observation which is also consistent with CTCs detected in patients with prostate cancer (Cho et al. 2012). Overall, relative morphometrics of CTCs have revealed fundamental insight on their biophysical properties and probable pathological origins.

8 Circulating Tumor Microemboli

Perhaps not surprisingly, circulating tumor microemboli (CTM or, equivalently, CTC aggregates) are detected with the HD-SCA workflow in a number of different studies across various cancer types, including breast, non-small-cell lung (NSCL), prostate, and pancreatic cancer (Marrinucci et al. 2012; Carlsson et al. 2014; Cho et al. 2012; Carlsson et al. 2017; Malihi et al. 2018). Intuition tells us that collective cell migration could be a travel strategy for distant metastasis (Friedl and Gilmour 2009; Friedl et al. 2012; Kats-Ugurlu et al. 2009). But how do we make this intuition precise? Early experiments to build clinical metrics for a physical understanding of the potential role of CTM in metastasis showed that individual CTCs within CTM were, on average, smaller in nuclear area (similar or equivalent to surrounding leukocytes) and length ($\sim$0.8-fold larger than surrounding leukocytes) than CTCs detected alone (where both nuclear area and length were $\sim$1.5-fold larger than surrounding leukocytes) (Marrinucci et al. 2012; Carlsson et al. 2014; Cho et al. 2012). Subsequent experiments exploited the HD-SCA assay's sensitivity and found that CTCs were present in a wide range for both early- and late-stage NSCL cancer (Nair et al. 2013). This led to the proposal that CTCs/CTM may be complementary to traditional clinical modalities for risk stratifying large lung nodules in patients and thus aid as a noninvasive diagnostic (Carlsson et al. 2014).

To test this idea, Carlsson et al. integrated CTC/CTM data with conventional clinical/imaging information to develop multiple logistic regression models using a case-control design in a training (N = 88 patients; N = 71 malignant; and N = 17 benign) and test (N = 41 patients; N = 33 malignant; and N = 8 benign) cohort (N = 129 total eligible patients); and then performed tenfold cross-validation of the entire group (Carlsson et al. 2014). Although the presence of CTCs and CTM is not always related to tumor burden or metabolic activity in the NSCL cancer setting, as measured by fluorodeoxyglucose (FDG) positron emission tomography-computed tomography (PET-CT) (Nair et al. 2013), Carlsson and colleagues demonstrated that patients could be diagnosed more accurately through CTM data combined with clinical/imaging information (where the area under the curve (AUC) = 0.88 and p value = 0.001 for all NSCL cancer patients, and AUC = 0.87 and p value = 0.002 for early-stage NSCL cancer patients), rather than clinical/imaging information alone (where AUC = 0.77 for all NSCL cancer patients as well as early-stage NSCL cancer patients).

Although it has been shown in mouse models that the metastatic potential of CTM is higher than single CTCs (Aceto et al. 2014), there are obvious open questions. Where do CTM come from, and where are they going? Are cells within CTM heterogeneous? What are the biophysical factors that allow CTM to endure the immune response in the circulatory system? Indeed, further technological improvements and multidisciplinary interactions are needed—and ongoing—to investigate the potential role of CTM in the bloodstream and organ arrest.

9 Expression-Level Measurements of CTCs

As mentioned at the start of this chapter, tumor heterogeneity and delineating which circulating cells are malignant across spatial regions in the body are a major challenge in cancer biology and oncology. We would like to know which biological signals in CTCs are relevant to disease progression and hence which CTC populations carry meaningful information about treatment response to ultimately provide clinicians with useful knowledge about the disease in real time. Toward this goal, Dago and coworkers sequentially characterized CTC subcellular AR expression fluctuations and clonal evolution in a patient with castrate-resistant prostate cancer (CRPC) as he progressed through chemotherapy and targeted therapy (Dago et al. 2014). CTCs from liquid biopsies were quantified (at both the phenotypic and the genomic scales) at 4 significant time points, where draw 1 was collected prior to any chemotherapy; draw 2 was collected after chemo- and radiotherapy; draw 3 was collected after 3 weeks of targeted therapy; and draw 4 was collected after 9 weeks of targeted therapy. The patient was showing clinical improvement at the time of draw 3, exhibiting less pain and lower prostate-specific antigen (PSA) levels; but, at the same time, a single clone, which strongly expressed AR, had also emerged. By draw 4, this treatment-resistant clone dominated the CTC population, and the patient exhibited increased pain and PSA levels. The patient died soon after.

Dago and colleagues showed that the dynamic changes across the genomic and phenotypic scales in CTCs, together with clinical information, allow for noninvasive monitoring of therapeutic efficacy. Recent work by Gross et al. examined rapid changes in CTCs immediately following bevacizumab (anti-angiogenic) treatment in CRPC: Blood samples were taken before and within 2 h after bevacizumab administration in 8 patients (Gross et al. 2015). In 6 of the 8 patients that responded, putative CTCs decreased, while apoptotic CTCs increased, suggesting early changes in tumor perfusion as well as which patients would likely benefit from bevacizumab therapy.

The fact that one can follow treatment sensitivity through repeated noninvasive biopsies opens the possibility of a very different approach to cancer treatment. In particular, if we can identify circulating biomarkers in the bloodstream, then we can *adapt* therapy to *control* the evolution of cancer in individual patients.

10 HD-SCA Beyond Fluorescence

Because all nucleated cells in a blood sample are assayed with the HD-SCA workflow, any cell of interest can be relocated on the glass slide to further probe the cell's physical, chemical, and molecular features (see Fig. 4). Such relocation has permitted the characterization of the mass (King et al. 2015; Phillips et al. 2012), volume (Phillips et al. 2012a; b), density (King et al. 2015; Phillips et al. 2012), density fluctuations (King et al. 2015; Phillips et al. 2012), and genome-wide copy number alterations (Dago et al. 2014; Ruiz et al. 2015) of individual disease-derived

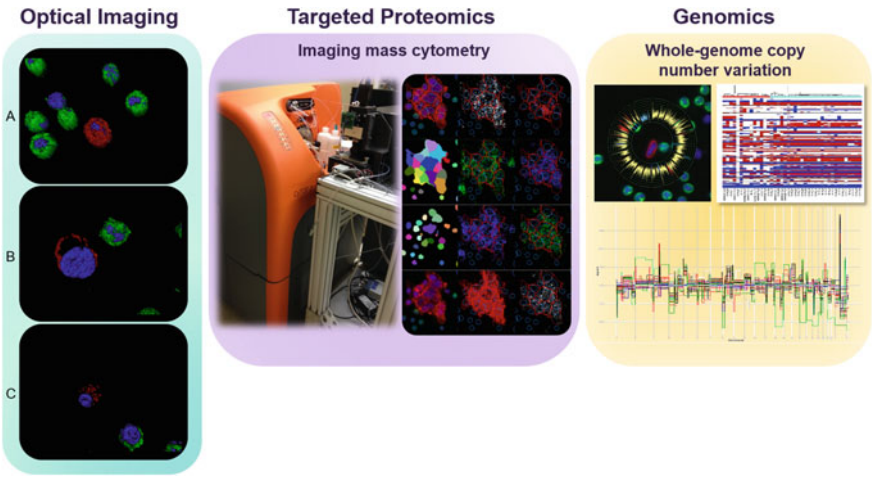

Fig. 4 Downstream characterization with the HD-SCA workflow Left panel: As an example of an optical imaging technique applied to detected cells of interest with the workflow, we present confocal images of **A** HD-CTC, **B** cross section of HD-CTC with membrane-bound cytokeratin, and **C** cross section of CTC-cfDNA producing displaying apoptotic blebbing. Here, images have been rendered with Imaris to visually define each cell and the expression of specific epitopes: Red represents CK expression, green represents CD45 expression, and blue represents DAPI. Middle panel: Targeted proteomic characterization on cells of interest using imaging mass cytometry. Right panel: Whole-genome copy number variation profiling of single cells of interest

cells. Dago et al., for example, developed a protocol for isolating DNA from CTCs under conditions suitable for downstream single-cell genomics. In this protocol, individual CTCs are identified, as described above, and subsequently picked off the slide with a micromanipulator for whole-genome amplification of their DNA, followed by library construction for Illumina sequencing. This method, as noted in the previous section, has led to the measurement of sequential clonal changes in CTCs in response to a multi-step therapeutic regime—which culminated in treatment resistance in the patient (Dago et al. 2014). Generalization of this method is straightforward and has been used to profile the copy numbers of CMCs in melanoma patients (Ruiz et al. 2015), thus furthering our ability to genomically characterize CTCs for the development of molecularly targeted therapies and monitoring patients.

In an effort to build (inputs for) blood cell flow models to better understand metastasis, Phillips and collaborators isolated cells of interest from liquid biopsies of patients with breast and ovarian cancer using the HD-SCA workflow and subsequently quantified the volume, density, and dry mass content through quantitative phase microscopy (Phillips et al. 2012a, b). In both cases, the average measured volume of CTCs was ($851 \pm 45.8\ \mu m^3$ for breast; $518.3 \pm 24.5\ \mu m^3$ for ovarian) greater than the leukocyte population ($234.1 \pm 4.1\ \mu m^3$ for breast; $230.9 \pm 78.5\ \mu m^3$ for ovarian). In the patient with ovarian cancer, the average dry mass content and density of CTCs were found to be 33.6 ± 3.2 pg and

0.065 ± 0.006 pg/fl, respectively. On the other hand, the average mass and density of the leukocyte population were, respectively, 18.7 ± 0.6 pg and 0.085 ± 0.004 pg/fl.

These imaging techniques have also been used to characterize CTM in transit (King et al. 2015). Again, using a combination of the HD-SCA workflow and quantitative phase microscopy, King et al. quantified the physical characteristics and subcellular density organization of CTCs and CTM in a patient with breast cancer. These measures were then used as a translational guide for in vitro and in silico models to investigate the mechanics of CTC transport in the vasculature. The in vitro model system was comprised of microfluidic flow assays to simulate tumor cell adhesion between breast cancer line CTCs/CTM and E-selectin under hemodynamic forces. These experiments found that CTCs exhibit an upward trend in rolling velocity as the number of CTCs/CTM increased, and similarly, the orthogonal displacement of CTM to the applied shear increased with CTM size. This observation was in accord with their numerical simulations of elastic collisions between CTCs and erythrocytes, suggesting that CTCs with more rigid membranes marginate quicker than those with softer membranes, and deformation of the membrane during collisions with erythrocytes can extend the time in which CTCs are flowing in the bloodstream. Taken together, these results provide a translational approach to formulate experiments in classical model systems and the design of numerical models that are grounded in real clinical metrics.

11 Conclusion and Outlook

By taking advantage of the HD-SCA workflow's simplicity and low-level processing of patient blood, intact CTCs can be identified, imaged, and further assayed at the phenotypic level down to the DNA polymer level. The high-throughput imaging workflow we have discussed above makes it clear that liquid biopsies present new opportunities for investigating and characterizing the spatiotemporal dynamics and clinical evolution of cancer. This effort becomes all the more tractable given modern technological developments in the analysis of circulating tumor DNA (Dawson et al. 2013; Forshew et al. 2012; Murtaza et al. 2013) and exosomal microRNAs (Taylor and Gercel-Taylor 2008) from fluid biopsies. In addition, Giesen and colleagues have recently introduced imaging mass cytometry—a combination of CyTOF mass cytometry, labeling of antigens in cells with rare-earth metal isotopes tagged to antibodies, and a high-resolution laser ablation system—for the simultaneous measurement of 32 proteins and posttranslational modifications at a cellular resolution of 1 μm (Giesen et al. 2014). This raises the possibility of extending the HD-SCA workflow for targeted proteomics and a system's biology approach to understanding the hematogenous dissemination of human cancer (Malihui et al. 2018; Gerdtsson et al. 2018). More concretely, it gives us a path to assess complex, hypothesized cellular states, such as the epithelial-to-mesenchymal transition (Thiery 2002), the mesenchymal-to-epithelial transition (Kalluri and

Weinberg 2009), cancer stem cells (Jordan et al. 2006), vasculogenic mimicry (Hendrix et al. 2003; Williamson et al. 2016), cell-to-cell interactions, cellular senescence (Hanahan and Weinberg 2011), tumor cell dormancy (Aguirre-Ghiso 2007), cancer cells in the blood and their pathological links to metastatic spreader or sponge sites (Newton et al. 2013, 2014), and other clinical relationships to (biomarker) expression dynamics. Now that many of these foundational steps have been solidified, we believe that the coming years will see dramatic progress in a comprehensive CTC assay as a real-time, noninvasive liquid biopsy for the development and implementation of patient-specific treatment strategies.

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Circulating Tumour Cells in Lung Cancer

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1 Introduction

Lung cancer is the second most commonly diagnosed cancer in both men and women and the leading cause of cancer-related death (American 2016). Lung cancers can be divided into two main cytological subgroups: small-cell lung cancer (SCLC), which represents ~20% of the cases, and non-small-cell lung cancer (NSCLC) representing the remaining ~80%. NSCLC is further subdivided into adenocarcinoma, squamous cell carcinoma and large cell carcinoma (~40%, ~25–30% and ~10–15%, respectively). Since around 70% of lung cancer patients present with advanced inoperable disease, chemotherapy and/or radiotherapy or targeted therapy are the most common treatment options (Hofman et al. 2016) and the 5-year survival rates are alarmingly low with (<7% for SCLC and <17% for NSCLC) (Schmidt-hansen et al. 2017). For the minority of patients who are present with early-stage NSCLC, surgery with curative intent is the most common therapeutic option; however, tumour recurrence still occurs in approximately 50% of the cases and overall survival remains still poor (Lee et al. 2013; Shaw et al. 2011). One potential contributing factor to poor survival rates in lung cancer is that diagnosis and choice of therapy are frequently based on the phenotypic and molecular characterisation of a single tumour biopsy, which may underestimate tumour heterogeneity and does not take into account tumour evolution with the ability to select for therapy resistance. Thus, it is now becoming extremely clear that monitoring of the disease is crucial for the success of the treatment. However, recurrent biopsies are generally not possible, are invasive and may not capture tumour areas that have acquired new driver mutations (de Bruin et al. 2014; Siegmund and

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Shibata 2016). Therefore, minimally invasive approaches that could improve early detection, identify early emerging of treatment resistance and bring to the discovery of new driver mutations are essential for optimising lung cancer care (Thiele et al. 2017; Perakis and Speicher 2017). The minimally invasive approaches, known collectively as "liquid biopsies", take advantage of the observation that the tumour can shed cellular and molecular components into the bloodstream where they can be identified as circulating tumour cells (CTCs) and circulating cell-free nucleic acids (cfNAs). Even though, in lung cancer, considerable advances have been achieved in examining cfNA, particularly circulating tumour DNA (ctDNA) (Abbosh et al. 2017; Offin et al. 2017), CTCs remain an important means of studying tumour biology since they can be used to establish in vivo and ex vivo models suitable for testing new drugs/drug combinations and identify possible mechanisms of resistance (Hodgkinson et al. 2014). Moreover, direct analysis of RNA and DNA from individual CTCs holds great promise to unravel intratumour heterogeneity and identify genes and signalling pathways relevant to therapeutic interventions (Klein et al. 2002).

To study CTCs, it is first necessary to find what may be one CTC among 10^6–10^7 white blood cells (WBCs) and consequently a wide range of CTCs methodologies have been developed as described in more detail elsewhere (Mohan et al. 2017), but they generally rely on three strategies: (American 2016) negative selection through removal of WBCs thereby enriching the remaining CTCs; (Hofman et al. 2016) positive selection of CTCs; and (Schmidt-hansen et al. 2017) analysis of all cells and then CTC identification through intensive image analysis. Each method presents its own advantages and weaknesses, and the choice of approach will depend on the clinical/biological aim of the study as well as pragmatic considerations such as cost and time available. Among the approaches based on positive selection, the most successful and widely used CTC technology is the FDA-recognised CellSearch® system (Janssen Diagnostics, Raritan, NJ, USA) which is a semi-automated platform that employs ferromagnetic beads coated with the epithelial cell surface marker, EpCAM, to enrich epithelial CTCs (Cristofanilli et al. 2004).

This review will focus on current CTC research in lung cancer; in particular, we will give an overview of CTC functional and molecular studies and will highlight findings on the potential use of CTCs in the clinical management of lung cancer.

2 Functional Studies on Lung CTCs

The improvements on CTC isolation methods make more attractive the possibility of functional analysis to better characterise the biology of CTCs and, in turn, further our understanding of both the primary tumour and the metastasis. CTC ex vivo cultures and in vivo tumour models offer the possibility of testing panels of known drugs with the aim of guiding patient therapy as well as providing screening platforms for new drugs. Establishing cell cultures or patient-derived xenograft

models from primary tumours or metastases is often difficult even when starting with millions of cells (Alix-Panabieres and Pantel 2014), so there is a considerable challenge in establishing the same types of models from the relatively low numbers of tumour cells present in the peripheral blood of cancer. However, in the last decade, several groups have established CTC cultures and in vivo models from cancer patients with advanced disease where CTCs numbers are high, especially when compared to limited stage disease (Hodgkinson et al. 2014; Yu et al. 2014; Kolostova et al. 2014).

In this section, we will emphasise the achievements made in the development of CTC pre-clinical models, their limitations and their use in lung cancer functional studies.

2.1 Ex Vivo Expansion of CTCs

There are a range of obstacles to be overcome when establishing CTC ex vivo cultures, and each step of the process from patient blood draw to expansion in culture has to be carefully implemented in order to avoid any CTC loss or death.

Firstly, as discussed above, not all cancer types and stages show a consistent number of CTCs in the peripheral blood and differences in CTC count are also observed in different subtypes within the same type of cancer (Kowalik et al. 2017). For example, patients with SCLC generally harbour ten times higher number of CTCs than patients with NSCLC (Hou et al. 2012). Another important aspect to be considered is that not all the CTCs retrieved in a blood draw will be viable as it has been shown that a large number of CTCs are apoptotic due to the loss of matrix-derived survival signals or circulatory shear stress (Larson et al. 2004). Moreover, it is possible that only a small population of CTCs has metastatic-initiating or growth potential, with the majority of CTCs residing in a quiescent state, which may inhibit ex vivo expansion (Zhang et al. 2013; Muller et al. 2005). Secondly, blood collection, transport to the laboratory and CTC enrichment must be compatible with CTC survival. It must be pointed out that there has been little systematic analysis of the effect of blood collection and transport (temperature and time) on ex vivo expansion and it is possible that specifically formulated blood collection tubes could be developed that would favour CTC survival. The third major obstacle is that optimal culture conditions for freshly isolated CTCs remain largely unknown. Although there are culture conditions that clearly do support CTC ex vivo expansion, it is difficult to know whether they allow all CTCs to grow or if they select for a subset of CTCs. Given this uncertainty, the lack of CTC expansion under a specific culture condition is not proof of lack of viable CTCs but only lack of CTCs that would respond to the culture environment.

Despite these obstacles, several groups have established CTC short-term and long-term ex vivo cultures in different cancer types such as breast, prostate and colon cancer, with success rates of 16%, 64% and 4%, respectively (Yu et al. 2014; Kolostova et al. 2014; Cayrefourcq et al. 2015) while to date studies reporting successful CTC cultures in lung cancer are limited. Nonetheless, recently a few

studies have succeeded in isolating and expanding CTCs in vitro from patients with lung cancer. For example, Zhang et al. developed a 3D co-culture model to expand CTCs isolated from 14 of 19 patients with early-stage lung cancer (Zhang et al. 2014). CTCs captured on a microfluidic chip were cultured with a mix of collagen, matrigel and cancer-associated fibroblasts (Zhang et al. 2014). Next-generation sequencing (NGS) revealed matched mutations, including *TP53* mutations, in patient tumours and CTCs which were not present in fibroblasts and healthy controls, confirming the tumour origin of the cultured cells (Zhang et al. 2014). This study highlighted the importance of the tumour microenvironment in establishing the expansion of early CTC ex vivo; however, this method is limited by the risk of fibroblast contamination that could affect further functional and molecular analyses. In another recent study, a microfluidic-based immunomagnetic approach was used to directly isolate CTCs from the blood of patients with lung adenocarcinoma and place them in a small volume of culture medium to achieve a high culture density (Wang et al. 2016). This approach increases the purity of starting CTC population but because of the lack of microenvironment stimuli, CTCs expand slowly (Wang et al. 2016). More recently, five CTC-derived cell lines have been generated from patients with recurrent SCLC (Klameth et al. 2017). These cell lines express typical SCLC markers such as synaptophysin, enolase-2 and chromogranin A, confirming their SCLC origin. Moreover, they spontaneously developed large multicellular aggregates with an increased resistance against chemotherapeutics commonly used for treatment of SCLC compared to the CTC single-cell suspension, suggesting that SCLC may rely on the formation of these aggregates with limited access for the drugs, lower growth fraction and hypoxic conditions (Klameth et al. 2017).

The generation of CTC ex vivo cultures represents an important step forward in the field of personalised treatment. Compared to the many months usually required for the generation of mouse tumour models, the time frame for ex vivo CTC cultures is shorter as seen in metastatic colon cancer where CTC cultures were established in less than a month (Grillet et al. 2017), which may eventually allow ex vivo CTC drug screening as a guide to therapy selection in the clinic. However, limitations of this approach still remain and an optimised protocol that is efficient, quick and applicable to all cancer types and stages is yet to be developed.

2.2 Circulating Tumour Cell-Derived Xenograft (CDX)

Recent studies showing that enriched CTCs from patient blood can form tumours in immunocompromised mice (Hodgkinson et al. 2014; Girotti et al. 2016; Baccelli et al. 2013) have directly confirmed that at least some CTCs are viable as well as tumourigenic with the resultant CDX representing valuable models for drug testing and understanding cancer development.

The first successful study establishing CDX models was performed in breast cancer where CTCs isolated from 110 patients were transplanted into the femoral medullary cavity of immunocompromised mice and three patient-derived CTCs were able to form multiple metastases (Baccelli et al. 2013). In SCLC, CTCs

enriched from chemo naïve patients who either later responded to chemotherapy (chemosensitive) or did not respond to chemotherapy (chemorefractory) were both capable of forming CDXs, whose response to platinum and etoposide treatment mirrored the response observed in the corresponding patients (Hodgkinson et al. 2014). From the same SCLC study, genomic analyses of single CellSearch® CTCs and CDX revealed that they are highly related in terms of both copy number and *TP53* and *RB1* mutations (Hodgkinson et al. 2014). This study also highlighted that establishing CDX models is a reliable and practical means of routinely generating SCLC xenografts where the availability of tumour tissue is often limited. It has also been shown in a case study that a CDX was obtained from a patient with NSCLC who lacked detectable EpCAM-/cytokeratin-positive CTCs (as measured by CellSearch®), indicating that the approach used may also enrich for viable CTCs with a more mesenchymal phenotype (Morrow et al. 2016). Molecular analysis of the resultant NSCLC CDX identified both mesenchymal and epithelial components in the tumour providing evidence that NSCLC mesenchymal CTCs are involved in the dissemination of the disease (Morrow et al. 2016). Circulating tumour cells were also shown to be tumourigenic in melanoma, where CDXs were established with a success rate of 13%, resembling patient tumours in terms of metastatic tropism and response to therapies (Girotti et al. 2016).

The advantages of CDX models compared to patient-derived xenograft (PDX) models include the ease of obtaining blood samples at several time points during the course of the disease, thus the possibility of longitudinal studies to investigate tumour evolution and mechanisms of drug resistance (Lallo et al. 2017). Moreover, given the fact that CDXs mirror the histopathology of the tumour donor (Hodgkinson et al. 2014), they represent a valuable alternative of tumour material in patients that cannot undergo surgery, as in the majority of SCLC cases. However, both ex vivo and in vivo expansions of CTCs run the risk that the resultant CDX or cultures are a result of selected expansion of a dominant clone that is best able to proliferate in that specific environment, and thus may underestimate and limit any study of tumour heterogeneity (Thiele et al. 2017). To determine the influence of either ex vivo or in vivo selection and identify the initial degree of CTC heterogeneity, it is important to also be able to carry out molecular analyses on enriched or single CTCs isolated directly from the patient blood. We will cover these recent advances that allow single CTC molecular analysis in the following section.

3 CTC Molecular Analysis

Molecular profiling of CTCs provides an exciting opportunity for adding valuable biological and clinical insights into cancer biology that may eventually benefit cancer patients. In addition to identifying potential drug targets, CTC molecular profiles can also be used to gain a better understanding of cancer progression. For example, as CTCs may be shed from both primary and metastatic sites, CTC molecular profiling could help identify their cellular origin, better understand the

tumour clonal kinetics and identify more effective drug targets. In early disease, it is possible to ask whether CTCs are shed randomly from all parts of the primary tumour or whether they originate from selected region(s). In addition, since CTCs can be routinely sampled longitudinally they can be used as a means of tracking tumour evolution and have been incorporated into the lung TRAcking non-small-cell lung Cancer Evolution through therapy [Rx] (TRACERx) study (Jamal-hanjani et al. 2014).

In this section, we will highlight the progress on CTC molecular profiling by mentioning initial studies on RNA and DNA extracted from the overall CTC population and ending with the most recent studies on single CTCs profiling. We will also discuss important initial findings from epigenetic and proteomic CTC analysis.

3.1 CTC RNA Profiling

A range of powerful single-cell RNA-Seq methods have emerged in the last five years and have been applied to CTC analysis (Navin 2015). Transcriptomic profiling of CTCs has the potential to shed light on the biology of the cells and identify activated pathways and potentially new drug targets. Utilising recent advances in CTC enrichment, several groups have examined RNA profiles from either CTC-enriched or CTC-depleted cell populations and compared them to RNA profiles from either healthy normal volunteers (HNVs) or total unenriched peripheral blood (Magbanua and Park 2014). Early observations clearly demonstrated that it is possible to detect specific epithelial markers, such as cytokeratin-19 (CK-19) in the peripheral blood of patients with cancer indicating the presence of CTCs (Stathopoulou et al. 2003). In NSCLC, the persistence of CK19 mRNA-positive CTCs after chemotherapy was associated with a poor clinical outcome (Milaki et al. 2017). Similarly, the detection of survivin mRNA from CTCs was linked to chemotherapy efficacy and survival for advanced NSCLC (Du et al. 2014). In an additional lung adenocarcinoma study employing reverse transcriptase quantitative real-time PCR (RT-qPCR), they found four candidate CTC-expressed genes (cytokeratin 7, Ca2+-activated chloride channel-2, hyaluronan-mediated motility receptor and the human telomerase catalytic subunit) were significantly elevated in patient whole peripheral blood mononuclear cells (PBMCs) compared to HNV PBMC controls (Man et al. 2014).

Since by its very nature RNA analysis of total PBMCs will be challenging given that the vast majority of cells sampled will be WBCs with CTCs comprising only a minor component, many recent studies have focussed on CTC-enriched samples. For example, a study using CellSearch® EpCAM-enriched CTCs from metastatic breast, prostate and colorectal cancer patients shows a significant increase of cancer-related genes detected compared to the unenriched fraction (Du et al. 2014). Subsequently, a CTC gene expression signature consisting of 35 genes was shown to be applicable in metastatic patients and HNVs (O'Hara et al. 2005). Using a similar type of approach, Sieuwerts and colleagues were able to examine RNA

expression in CellSearch®-enriched CTCs from patients with metastatic breast cancer and were able to identify CTC-specific expression patterns that correlated with CTC numbers (Sieuwerts et al. 2009).

However, not all the CTC enrichment methods are compatible with RNA analysis, especially when a preservative is added in the blood tube which may impact negatively on the RNA quality. For this reason, the development of microfluidic devices that provide the advantage of the use of unfixed blood is the best option to maximise RNA quality for expression profiling. Recent studies, using CTC-iChip microfluidic device, performed RNA sequencing of CTCs isolated from patients with prostate, breast and pancreatic cancers, and they identified increased expression of Wnt signalling pathways in a subset of cells (Miyamoto et al. 2015; Aceto et al. 2014; Ting et al. 2015). Moreover, single-cell RNA profiling was performed on CTCs from lung, breast and prostate cancers revealing a consistent induction of β-globin (HBB) which contributes to their ability to survive in the bloodstream and initiate distant metastases. HBB was not expressed in the corresponding primary tumours and metastatic sites suggesting that it is a unique feature that cells acquire in the vasculature (Zheng et al. 2017). A recent study developed a digital quantitation of RNA from metastatic prostate CTCs enriched by microfluidic device and showed that the detection of prostatic aberrant RNA transcripts is able to identify patients resistant to targeted therapy, hence highlighting the potential role of CTC RNA analysis in the prediction of patient clinical outcome (Miyamoto et al. 2018).

These important findings, showing unique biological traits of CTCs in different cancer types, would not be possible without the development of technologies that now enable the efficient isolation of CTCs with intact RNA, and the application of single-cell RNA sequencing strategies.

3.2 CTC DNA Profiling

The increases in the sensitivity of DNA technologies have been pivotal for CTC research since they have enabled researchers to confirm the tumour identity of cells identified by establishing that they harbour tumour-specific molecular signatures (Heitzer et al. 2013; Lohr et al. 2014; Klein et al. 1999). Since DNA is more stable than RNA, genomic analysis can be readily applied to CTCs obtained from preserved blood where RNA is heavily degraded. Since copy number changes are common in cancer cells and rare in non-cancer cells analysis of copy number alterations (CNA) can provide a simple but powerful means of confirming the tumour origin of CTCs identified by antibody staining (Magbanua and Park 2014). For single CTC analysis, following an initial CTC enrichment, an additional identification/isolation step is required and can be achieved in a variety of ways including fluorescence-activated cell sorting (FACS) (Swennenhuis et al. 2013), CTC visualisation followed by isolation via micromanipulation (Chen et al. 2013) and the DEPArray™ automated system which incorporates cell manipulation via electrostatic charge (Peeters et al. 2013).

Once isolated a single cell will typically contain on average only two copies of each gene amounting to six picograms of DNA necessitating whole-genome amplification (WGA) prior to detailed profiling. Several single-cell WGA techniques have been described which can largely be divided into three main categories: (American 2016) direct PCR amplification; (Hofman et al. 2016) multiple displacement amplification (MDA); and (Schmidt-hansen et al. 2017) multiple annealing and looping-based amplification cycles (MALBAC®). These methods have been extensively described elsewhere (Gawad et al. 2016), but briefly the PCR-based WGA methods are based on the fragmentation of the single-cell genome and ligation of adapters to the resulting sticky ends followed by exponential temperature cycled amplification (Spits et al. 2006). The *Ampli*1™ kit from Menarini Silicon Biosystems is an example of this category (Klein et al. 1999). The MDA method uses random priming following by isothermal amplification using a high-fidelity polymerase with a strand displacement activity (Dean et al. 2002). REPLI-g (QIAGEN, Hilden, Germany) is one of the commercially available kits based on the MDA method. Finally, MALBAC® is a patented hybrid method with an initial isothermal amplification in which common sequences are added, followed by PCR amplification (Zong et al. 2012), and Yikon Genomics now has a commercial kit based on this technology. All the three methods mentioned have advantages and disadvantages, and the choice to use one or another depends on which downstream analysis to be performed.

The main technical advantage of single CTC analysis is that it excludes any complications due to the presence of contaminating cells. Additionally, since each cell typically has around two copies of each gene the depth of NGS required is low compared to the sequencing depth needed for the analysis of complex tissues. Moreover, single-cell data provides an ideal approach to establish the degree of tumour heterogeneity and estimating tumour evolution. For example, a study of Heitzer et al. showed the potential of copy number and mutation analysis by applying NGS and array-comparative genomic hybridisation (aCGH) technologies on CTCs isolated from patients with metastatic colorectal carcinoma (Heitzer et al. 2013). Although major CNA and driver mutations observed in CTCs were shared with matching primary tumours and metastatic lesions, private genomic alterations were also detected in single CTCs (Heitzer et al. 2013). This study highlighted the importance of analysing CTCs at the single-cell level, because bulk analysis may lose important information on tumour heterogeneity with subsequent impact for patient clinical management. In another study, whole-genome amplification performed on CTCs from 11 patients with lung cancer, and single-cell whole-exome sequencing data identified characteristic cancer-associated mutations, providing information potentially useful for the clinic like drug resistance and phenotypic transition (Ni et al. 2013). In contrast to mutational analysis which revealed extreme cell-to-cell heterogeneity, genome-wide CNA patterns were largely similar in each CTC from each patient (Ni et al. 2013). In a recent study, genome-wide CNA profiles were generated on individual CTCs from SCLC and common regions of gain (3q, 5p) and loss (3p, 17p) were identified across patients. Moreover, CNA patterns detectable in single and pooled CTCs from 31 pre-treatment patients could

classify patients as either chemosensitive or chemorefractory to standard cisplatin–etoposide with an accuracy of 83.3% (Carter et al. 2016).

Given the ease of blood sampling, CTC analyses have the potential to replace traditional tissue biopsy particularly in the metastatic setting, where tissue biopsies are often limited by procedural risks associated with the lesion site and patient physical conditions. In metastatic breast cancer, CNA and somatic mutations were found to be 85% concordant when comparing paired single CTCs and metastatic tissue (Paoletti et al. 2018). However, private genetic alterations were found in either CTCs or metastasis, highlighting the need for further investigations aiming to address if this discordance is biological or purely technical (Paoletti et al. 2018).

Detailed de novo mutational detection following single WGA is severely hampered by polymerase misincorporation rates affecting both WGA and NGS steps as well as damage to the DNA encountered during either isolation or WGA such as the recently described cytosine deamination caused by DNA denaturation at elevated temperatures (Dong et al. 2017).

Finally, the integration of RNA and DNA analysis, shown to be possible in single cells (Klein et al. 2002), can establish the linkage between the genetic alterations and activation of RNA pathways, helping to better understand CTC biology, metastatic process and mechanism of drug resistance.

3.3 CTC Methylation Profiling

Besides transcriptomic and genomic alterations, lung cancer is characterised also by epigenetic abnormalities and recent studies on lung cancer epigenetics have revealed promising biomarkers, particularly involving changes in DNA methylation. In particular in NSCLC, hypermethylation patterns are associated with cigarette smoking (Sato et al. 2014) and clinical parameters (Walter et al. 2012; Brock et al. 2008). Methylation of cytosine–guanine dinucleotides (CpG) at cytosines is a major epigenetic event contributing to genome organisation and proper gene expression (Jones 2012). The detection of DNA methylation can be performed by methods relying on bisulphite conversion of unmethylated cytosines to uracils, while it does not significantly react with methylated cytosines. Analysis of the sequence obtained after conversion allows to discriminate uracils from cytosines and to infer the methylation status of the original DNA. Considering the important role of DNA methylation in cancer, recent studies have shown how it is possible to monitor methylation changes in single cells. For example, Smallwood et al. demonstrate in mouse embryonic stem cells that large-scale single-cell epigenetic analysis is achievable, and demonstrate that single-cell bisulphite sequencing is a powerful approach to accurately measure DNA methylation at cytosine residues across genomes of single cells (Smallwood Sa et al. 2014).

However, methylation analysis of CTCs is a field not entirely explored yet, which could be attributed to the technical challenges associated with studying DNA methylation in single cells (Walter et al. 2012). Nonetheless, some groups have described successful methods for investigating methylation patterns in CTCs such

as use of single-cell agarose-embedded bisulphite sequencing (scAEBS) (Pixberg et al. 2017) which showed clear differences between CTCs and WBCs as well as demonstrating that hypermethylation at promoters of key EMT genes is an infrequent event in CTCs (Pixberg et al. 2017). In breast cancer, methylation of SOX17 (a tumour suppressor) was examined in primary tumours, corresponding CTCs and ctDNA of 153 patients with cancer and HNVs. The study showed a direct association between SOX17 promoter methylation in CTCs and ctDNA in patients with both early and metastatic breast cancer; in contrast, there was no correlation between the methylation status of the primary tumour and the corresponding CTC and ctDNA indicating that the tumour cells that become CTCs and contribute to ctDNA may represent a small subclone of the primary cancer (Chimonidou et al. 2017). While CTC methylation has been investigated in breast, prostate and colorectal cancer, there have been no reports of similar studies in lung cancer yet. However, a recent study established a combined strategy where enriched CTCs from patients with lung cancer were processed in one tube followed by liquid chromatography−electrospray ionisation−tandem mass spectrometry (LC-ESI-MS/MS) analysis. Using this approach, they showed for the first time significant changes of DNA and RNA methylation in CTCs compared with whole blood cells (Lu et al. 2017).

The study of epigenetic mechanisms happening in CTCs, coupled with DNA and RNA molecular analysis, represents a valuable approach to study cancer biology and has the potential to identify new biomarkers for monitoring cancer progression and therapeutic response.

3.4 CTC Protein Expression Profiling

In contrast to the fields of genomic and transcriptomic analysis, comprehensive proteomic profiling of CTCs is relatively unexplored largely because the quantification of proteins at a single cell level without an appropriate amplification is very challenging. Nonetheless, Zhang et al. reported a microchip-based approach for the co-detection of intracellular proteins, glucose uptake as well as genetic mutations at a single-cell level (Zhang et al. 2015). Using this approach, intracellular proteins from thousands of individual CTCs can be quantified using antibody barcoding while, simultaneously, glucose uptake can be measured via fluorescent imaging and CTC cell nuclei can be retrieved for subsequent genomic analysis. In a pilot study, CTCs from a patient with NSCLC were examined and it could be shown that for more than 80% of the CTCs there was successful measurement of eight intracellular proteins (Zhang et al. 2015). Despite measuring only a small proportion of proteome, the method described has an important impact on CTC global characterisation as it integrates information of the proteomic, metabolic and genetic changes in a single cell. Another recent study described the development of a microfluidic assay to measure multiple protein targets in single CTCs and its application to CTCs from 12 patients with breast cancer (Sinkala et al. 2017). The method is called rare-cell, single-cell western blot (scWB), and it couples PAGE of

single-CTC lysate with subsequent antibody probing of PAGE-resolved protein targets (Sinkala et al. 2017). By using a label-free CTC enrichment platform that utilises cell size and deformability, they were able to use a protein panel comprising of specific targets for breast cancer, epithelial markers and housekeeper proteins to distinguish CTCs from WBCs. Although this method needs further confirmation with a larger cohort of patients, the targeted proteomic methodology is a promising approach for identifying new CTC targets of interest (Sinkala et al. 2017).

4 CTC Lung Clinical Studies

While molecular and genetic characterisation of CTCs is a relatively new area which is in the process of being translated to the clinic, CTCs enumeration has been widely used to guide prognosis in patient with metastatic disease, to monitor treatment response, to identify patients requiring adjuvant therapy in early stage or, in surveillance, to detect relapsing disease (O'Flaherty et al. 2012). In this section, we will discuss a range of potential uses for CTCs in the clinic with a major emphasis on lung cancer.

4.1 CTCs as Biomarkers for Early Detection

Since primary tumours can shed CTCs throughout their development even at early stages of tumour development (Rhim et al. 2012), sensitive methods for the detection of CTCs may provide a sensitive approach for early cancer detection. Early detection would be particularly beneficial for patients with lung cancer where the bulk of diagnosis is at late-stage cancer where treatment with curative intent is not feasible. In a promising example, it was shown that the detection of CTCs in five out of 168 chronic obstructive pulmonary disease patients predicted occurrence of lung nodules 1–4 years after CTC detection (Ilie et al. 2014). However, in the same study there was also a false-positive rate since three patients who harboured detectable CTCs did not develop overt cancer. More recently, a study evaluated the presence of CTCs to differentiate benign from malignant lung lesions (Fiorelli et al. 2015). CTCs were detected in 90% of patients with malignant lesions, while with patients harbouring benign lesions, CTCs were detected in 5% of the cases as well suggesting that detection of CTCs may be a valid marker in the early detection of lung cancer (Fiorelli et al. 2015). Early diagnosis of cancer by detection of CTCs is appealing since a simple blood draw could be readily included in routine health screen. However, because of the challenges due to the low numbers of CTCs involved and potentially high false positives the feasibility of using CTCs in early lung cancer detection needs a large well-controlled trial.

4.2 CTCs as Prognostic Biomarkers

Prognostic biomarkers are indicators of the patient's overall clinical outcome including progression-free survival (PFS) and overall survival (OS) (Nalejska et al. 2014). As CTCs are thought to be responsible for metastasis, many groups have explored their potential use as prognostic markers.

The first observation about the CTC prognostic value was back in 2004 when CTCs from patients with metastatic breast cancer were enumerated using the CellSearch® system showed that they were associated with progression-free survival (PFS) and overall survival (OS) (Cristofanilli et al. 2004). Since then, the US Food and Drug Administration (FDA) approved CellSearch® as a method with prognostic utility in breast cancer using a cut-off of five cells per 7.5 ml. The FDA approval of CellSearch® was then extended to prostate cancer (de Bono et al. 2008) and colorectal cancer (Cohen et al. 2008), with a cut-off of five and three CTCs per 7.5 ml of blood, respectively.

Several groups have also demonstrated the prognostic utility of CellSearch® CTCs in lung cancer. In particular, Krebs et al. reported that CellSearch® detection of five CTCs (per 7.5 ml of blood) in patients with advanced NSCLC is a poor prognostic factor and that a change in CTC number after a single cycle of standard-of-care chemotherapy predicts survival outcome (Krebs et al. 2011). In SCLC, which is one of the solid tumours where the CTCs are more abundant than any other solid tumour thus far reported with a range of 0–44,896 cells per 7.5 ml blood, the corresponding CellSearch® CTC count cut-off is set at a higher value of 50 CTCs per 7.5 ml blood (Hou et al. 2012). However, CellSearch® presents some limits such as the detection of CTCs in some healthy individuals or patients with inflammatory disease (Allard et al. 2005). Moreover, CellSearch® enriches for EpCAM-positive CTCs thus excluding mesenchymal CTCs or stem cell-like CTCs with low or absent expression of epithelial markers, while they probably play a crucial role in cancer metastasis and drug resistance. For these reasons, other CTC detection methods have been used to evaluate the prognostic significance of CTCs. A NSCLC study using the ISET® CTC technology (CTCs enriched based on cell size) examined blood from 208 patients with stages I-IV identified CTCs in 50% of patients (Hofman et al. 2011). Although this study found no correlation between the numbers of CTCs and the staging of the disease, it concluded that a threshold of >50 CTCs corresponded to shorter PFS and OS (Hofman et al. 2011). However, the CellSearch® CTC study by Krebs et al. (above) showed a threshold of five CTCs in 7.5 ml blood in 101 stage III/IV NSCLC patients related to poor prognosis (Krebs et al. 2011) highlighting the need for standardisation of CTC detection methods.

In early-stage lung cancer, the number of CTCs detected in the peripheral blood using CellSearch® is lower compared to SCLC (Hou et al. 2012). For this reason, pulmonary vein sampling was considered for its proximity to primary tumour that implies that it may be advantageous to improve the sensitivity of CTC detection. A pilot study performed by Crosbie et al. compared blood sampling from peripheral and tumour-draining pulmonary veins for CTC detection using CellSearch® at the

time of tumour resection (Crosbie et al. 2016). Significantly, more CTCs were detected in pulmonary vein blood than in matched peripheral blood, and the presence of pulmonary vein CTCs was an independent risk factor for lung cancer recurrence and death (Crosbie et al. 2016).

Other groups have evaluated the clinical relevance of CTCs in NSCLC by analysing CTC protein expression. One such study examined the individual CTC composition in patients with NSCLC receiving platinum-based treatment (Nel et al. 2014). In particular, they stained CTCs for both epithelial markers such as EpCAM and pan-cytokeratin and mesenchymal markers such as N-cadherin and CD133 (Nel et al. 2014). They identified different subsets of CTC populations with heterogeneous combinations of epithelial and mesenchymal characteristics. Moreover, the presence of mesenchymal markers predicted shorter PFS (2 vs. 8 months, $P = 0.003$), showing an important role of the mesenchymal CTC population in the emergency of drug resistance (Nel et al. 2014).

However, the prognostic role of mesenchymal CTCs has not been fully established yet with conflicting results reported. For example, a pilot study performed on 27 patients with metastatic lung cancer showed that the presence of EpCAM-positive epithelial CTCs was associated with poor outcome, whereas the EpCAM-negative mesenchymal CTC was not (Wit et al. 2015). Thus, the difference between EpCAM-positive and EpCAM-negative CTCs as prognostic markers needs to be further investigated.

The prognostic relevance of CTC clusters or circulating tumour emboli (CTMs) has also been discussed in many studies. For example, CTMs were detected by ISET® technology in 43% of patients with stage IIIB/IV NSCLC (Krebs et al. 2012), and in an independent study using high-definition CTC assays, CTMs were seen in 50% of patients with stage I-IV NSCLC (Carlsson et al. 2014). Both studies highlighted the prognostic value of CTMs. Furthermore, analysis of pulmonary vein blood in patients with NSCLC also revealed the existence of CTCs and CTMs in 33% of patients which predicted tumour recurrence and poorer disease-free survival (Crosbie et al. 2016).

In conclusion, several studies have shown the prognostic utility of CTCs in lung cancer which holds great promise to be routinely used as prognostic markers in the clinic. However, there are many limits that need to be solved, as the use of more sensitive technologies that allow isolation and accurate characterisation of CTCs in larger sample sizes.

4.3 CTCs as Predictive and Pharmacodynamic Markers

Analysis of CTCs may also provide information on the likelihood of response to specific therapies, thereby facilitating the selection of more effective personalised therapies. An early pioneering study investigated EGFR mutations in CTCs from 12 patients with metastatic NSCLC known to harbour EGFR mutations; the investigators were able to detect matching EGFR mutations, including the resistance mutation T790 M, in CTCs from 11 patients (Maheswaran et al. 2008). These

results were confirmed also in another study where EGFR mutations were detected in 84% of 37 patients carrying EGFR-mutant primary tumours (Marchetti et al. 2014). Only 6% of the EGFR mutations found in the CTCs did not match the mutations in the primary tumour which may reflect tumour heterogeneity (Marchetti et al. 2014). Punnose et al. also analysed CTCs for EGFR mutational status by quantitative PCR, and they observed that mutational status in CTCs was concordant with tumour biopsies (Punnoose et al. 2012). Taken together, these findings indicate that molecular profiling of CTCs could be used for target identification and selecting personalised therapies and may be incorporated into the clinical management of lung cancer. In a more recent study, investigating the EGFR mutational status in serially sampled CTCs and ctDNA in patients with NSCLC showed that that early detection of critical drug-resistant mutations, such as EGFR T790 M, was associated with worse survival outcome (He et al. 2017).

In addition to mutational analysis, genome-wide CNA analysis offers a powerful means to perform CTC profiling that can also be used to predict cancer progression as well as emergence of secondary resistant mechanisms. Ni et al. showed that CTCs obtained from 11 patients with lung cancer exhibited reproducible CNA patterns more closely corresponding to the metastatic tumour than the primary (Ni et al. 2013). Moreover, in a recent SCLC study a biomarker was developed based on CNA patterns detectable in single and pooled CTCs from 31 pre-treatment patients and was able to classify patients as either chemosensitive or chemorefractory to standard cisplatin etoposide with an accuracy of 83.3% (Carter et al. 2016). Interestingly, in the same study five patients who responded to treatment and relapsed with chemorefractory disease showed unaltered CTC CNA patterns at relapse indicating that mechanism of acquired chemoresistance may differ from de novo chemoresistance (Carter et al. 2016).

Rearrangements or translocations of *ALK* and *ROS1* genes have been also examined in CTCs to evaluate their potential utility in guiding therapy (Pailler et al. 2013; Faugeroux et al. 2014). In a recent study, aberrant ALK patterns were examined using fluorescence in-situ hybridisation (FISH) in CTCs collected at initiation of crizotinib therapy from 39 patients with NSCLC carrying ALK rearrangements or ALK copy number gain. Baseline CTC count was not predictive of crizotinib benefit, while a significant association between the dynamic evolution of the numbers of CTCs carrying ALK copy number gain and median PFS was found in 29 patients monitored at an early time point of crizotinib. These results highlight the potential use of CTCs as predictive biomarker of early progression on crizotinib treatment in patients with ALK-rearranged NSCLC (Pailler et al. 2017). A similar approach was also used to detect both ROS1 translocations and CNA in NSCLC CTCs which correlated with poor response to crizotinib treatment (Pailler et al. 2015).

The change in the numbers of CTCs or a particular subset of CTCs following surgery, radiation and chemotherapy may be linked to response to therapy which makes CTCs potential pharmacodynamic prognostic markers. For example, in SCLC, 51 patients starting chemotherapy or chemoradiotherapy were enrolled and CTCs were enumerated using CellSearch® at baseline, after chemotherapy, and at

relapse (Naito et al. 2012). They observed that patients whose CTC count remains ≥ 8 CTC/7.5 ml post-treatment show a worse overall survival than those whose CTC levels dropped down (Naito et al. 2012). Similar results were also observed in patients with NSCLC showing that >2 CTCs/7.5 ml or any increase in CTC numbers after therapy predicted lower OS and PFS (Muinelo-Romay et al. 2014; Juan et al. 2014). Changes in CTC counts were also examined in patients with localised NSCLC undergoing radiation treatment, and the change in CTC counts post-treatment was evaluated as a surrogate for disease response (Dorsey et al. 2015). In this study, CTCs were detected in 65% of patients prior to the start of radiotherapy and on completion of radiotherapy CTC counts were below the threshold in all but one patient who later developed metastatic disease post-completion of radiotherapy (Dorsey et al. 2015).

Interestingly, in a study where NSCLC patients were undergoing treatment with the immune checkpoint blockade drug nivolumab it was shown that both expressions of the drug target (anti-programmed cell death ligand 1, PD-L1) in CTCs and CTCs numbers were found to be associated to poor outcome suggesting CTCs may prove useful in selecting immune-based therapies (Nicolazzo et al. 2016).

In summary, CTC analyses could provide relevant information for personalised therapies, as they could allow a real-time monitoring of changes in the genomic landscape of the tumour that are responsible for the appearance of drug-resistant clones.

5 Conclusions

Applications of CTCs as "liquid biopsy" have dramatically increased in oncology in the last years, establishing their clinical impact as biomarkers for early detection, prognostic and pharmacodynamics markers. Recently, sequencing at single-cell resolution is now possible which may represent an extraordinary opportunity to characterise the mutational profile of CTCs, to interrogate tumour heterogeneity and evolution with longitudinal samples, as well as to dissect fundamental pathways that are involved in the metastatic process. Moreover, it has been shown that CTCs can be expanded in culture or in mice providing an evaluable tool for drug testing and models to study CTC biology. In addition to CTC analysis, the combination with matched ctDNA, now possible from the same preserved blood tube (Chudziak et al. 2016; Rothwell et al. 2015), provides an exciting opportunity to understand the patient's disease cancer status.

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Circulating Tumor Cells in Breast Cancer

Diana H. Liang, Carolyn Hall and Anthony Lucci

1 Introduction

Breast cancer remains the most frequently diagnosed malignancy among women, accounting for nearly 30% of all new cancer diagnoses in women in the USA (DeSantis et al. 2011; Siegel et al. 2017). Due to early detection and improvements in treatment, breast cancer mortality has decreased steadily over the past two decades (Siegel et al. 2017). However, gaining further understanding of breast cancer metastasis is necessary, as 25% of non-metastatic breast cancer patients will develop distant metastases after initially successful treatment (Hall et al. 2016). Furthermore, 10–50% of patients with negative axillary lymph nodes at the time of curative surgery later develop distant metastasis (Green and Hortobagyi 2002; Fisher et al. 1983; Gilbey et al. 2004). Together, these data suggest that bloodstream tumor cell dissemination (circulating tumor cells, CTCs) and distant micrometastases that are undetectable by currently available diagnostic tools can develop during the early stages of breast cancer progression. CTCs have been identified as the potential source of micrometastases responsible for treatment failures and have become an active area of translational cancer research.

CTCs are rare, phenotypically diverse cell populations with varying viability, dormancy, biomarker expression, and metastatic potentials (Hall et al. 2016). Since CTCs numbers are extremely low compared to white blood cells (Hughes et al. 2012), isolation of CTCs from patients' blood samples has been a challenging process; therefore, considerable translational research efforts have been dedicated to development of sensitive and specific assays to identify and isolate them.

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Over the last decade, a rapidly growing body of the literature has shown promising clinical relevance of CTCs in non-metastatic as well as metastatic breast cancer patients. CTCs are detected in 10–80% of breast cancer patients, depending on the CTC detection method used and stage of the disease (Banys et al. 2012). While CTC detection rate is considerably higher in metastatic cases, the prognostic potential of CTCs has been demonstrated in both non-metastatic and metastatic breast cancer cases (Hall et al. 2016; Banys et al. 2012; Banys-Paluchowski et al. 2016). Furthermore, CTCs have been shown to be potentially useful as markers to monitor response to cancer therapies as well as to guide targeted therapies (Hall et al. 2016; Banys et al. 2012; Banys-Paluchowski et al. 2016; Balic et al. 2012). As a form of "liquid biopsy," CTCs may serve as an alternative to invasive tissue biopsy. Both intratumor heterogeneity and tumor heterogeneity between primary tumor and metastatic lesions are now well-accepted phenomena (Navin et al. 2011; Ma et al. 2012). However, as CTCs are derived from the primary tumor as well as from metastatic sites, they may be more comprehensive surrogates for the genetic characterization of systemic disease. Molecular characterization of CTCs has the potential to allow clinicians to better select individualized cancer therapies for patients and to allow real-time monitoring for resistance to anticancer therapies (Nadal et al. 2013). Here, we will summarize the clinical significance of CTCs in breast cancer patients and will discuss the potential for and barriers to more widespread application of CTCs in clinical care.

2 Disseminated Tumor Cell Identification

2.1 Bone Marrow Micrometastasis in Breast Cancer

Since the identification of micrometastatic disease in the bone marrow of early breast cancer patients by immunocytochemistry in 1980 (Sloane et al. 1980), the presence of these disseminated tumor cells (DTCs) has been associated with poor outcomes overall (Mansi et al. 1987, 1991, 1999, 2016; Braun et al. 2005; Hall et al. 2012). In the largest published study to date, Braun et al. reported that 30.6% of 4703 stage I–III breast cancer patients had bone marrow micrometastasis (DTCs) at the time of their initial diagnosis (Braun et al. 2005). Patient outcomes over a 10-year follow-up period were investigated. In the study, the authors found that the patients with bone marrow micrometastatic disease tended to have larger tumors, tumors with higher histologic grade, more lymph node involvement, and hormone receptor-negative disease. Furthermore, the presence of DTCs was a significant prognostic factor for poor disease-free survival, overall survival, as well as breast-cancer-specific survival. In multivariable analysis, the presence of DTCs was an independent predictor of worse outcome and was associated with higher risk of death from any cause, death from breast cancer, local recurrence, and distant metastasis at 5 years.

In another study of 5210 patients with clinical T1-2N0M0 breast cancer who were enrolled in the American College of Surgeons Oncology Group Z0010 trial, the detection rate of DTCs in the 3413 bone marrow specimens examined by immunocytochemistry was lower, 3.0%; yet the presence of DTCs was associated with decreased overall survival (Giuliano et al. 2011). Interestingly, there was no concordance between the presence of occult metastases in sentinel lymph nodes and the presence in the bone marrow, and the presence of sentinel lymph node metastases was not significantly associated with overall survival (Giuliano et al. 2011). There was, however, an association between increasing tumor size and sentinel lymph node metastases, but no significant relationship between tumor size and the rate of microscopic bone marrow disease.

2.2 Circulating Tumor Cell Detection: CellSearch® System

Drawing blood for circulating tumor cells (CTCs) is an alternative to performing an invasive bone marrow biopsy to detect DTCs. Currently, the only US FDA-approved method for detection of CTCs is the CellSearch® System (Menarini Silicon Biosystems, Italy) (Balic et al. 2012). This automated system for detection of CTCs has been shown to be reproducible across different independent testing sites and has been FDA-approved for prognostic evaluation and therapeutic response monitoring in patients with metastatic breast, prostate, or colon cancer (Balic et al. 2012; Cristofanilli et al. 2004, 2005; Allard et al. 2004). CellSearch® uses an immunomagnetic enrichment process to evaluate a 7.5-mL sample of blood, based on positive selection with epithelial cell-specific EpCAM-labeled iron oxide nanoparticles and subsequent detection of cytokeratin (CK 8, 18, 19)-positive CTCs (Balic et al. 2012; Allard et al. 2004; Krawczyk et al. 2014). This CellSearch® process further selects out cells that lack CD45 expression and have the cytomorphologic characteristics of tumor cells, based on size and morphology, presence of a viable nucleus, and an appropriate nuclear-to-cytoplasmic ratio (Balic et al. 2012; Allard et al. 2004). The resulting CTC enumeration is reported as the number of CTCs per 7.5-ml blood (Allard et al. 2004).

In the initial 2004 study utilizing the CellSearch® System, CTCs were enumerated in 72 healthy premenopausal women, 73 healthy postmenopausal women, 199 women with benign breast diseases or other non-malignant diseases, and 422 patients with metastatic breast cancer (Allard et al. 2004). In healthy patient blood samples, only 5.5% had 1 CTC per 7.5 mL of blood with no sample with 2 or more CTCs per 7.5 ml of blood. Similarly, only 7.5% of patients with benign breast diseases or other non-malignant diseases had 1 CTC in 7.5-mL blood sample. The mean number of CTCs with women without known malignancy was only 0.1 ± 0.2 CTC per 7.5 mL of blood. In the metastatic breast cancer population, the mean number of CTCs was 84 ± 885 per 7.5 mL of blood. Thirty-seven percentage of metastatic breast cancer patients had 2 or more CTCs, with 10% having 50 or more

CTCs per 7.5 mL of blood. Since the reporting of this study, which demonstrated that the CellSearch® System provides an accurate and reproducible assay, many studies have applied this system to study its potential clinical utility, which we will review in this chapter.

3 Circulating Tumor Cells in Metastatic Breast Cancer Patients

3.1 Prognostic Value of CTC Counts in Metastatic Breast Cancer

Using the CellSearch® System described above, Cristofanilli et al. conducted a prospective double-blind study at 20 clinical centers throughout the USA to evaluate the utility of CTC-level measurements in predicting responses to therapy, progression-free survival, and overall survival in 177 metastatic breast cancer patients (Cristofanilli et al. 2004). In this study, patients underwent standard imaging studies to evaluate metastatic lesions and had blood samples collected to enumerate CTCs prior to starting a new treatment. Disease statuses in these patients were reevaluated every 9–12 weeks. This study also demonstrated that CTCs were rare in healthy women and in women with benign breast disease. While none of the healthy control subjects had 2 or more CTCs per 7.5 ml of blood, 61% of the patients with metastatic breast cancer had 2 or more CTCs per 7.5 ml of blood. To select the number of CTCs that most clearly separates patients with rapid disease progression from those with slow progression, the authors systemically correlated thresholds of 1 to 10,000 CTCs at patients' baseline levels with their rates of progression-free survival. The authors found that the median progression-free survival among patients reached a plateau at approximately 5 cells per 7.5 mL of blood. Therefore, outcomes were compared between patients who had less than 5 CTCs per 7.5 mL of blood prior to initiation of new treatment for metastatic disease and patients who had 5 or more CTCs per 7.5 mL of blood. The median progression-free survival for all 177 patients in the study was 5.0 months, and the median overall survival was more than 18 months. Of these 177 patients, 49% had 5 or more CTCs at baseline prior to initiation of new therapy. This group of patients had a significantly shorter median progression-free survival of 2.7 months and median overall survival of 10.1 months, when compared to the group of patients with less than 5 CTCs, who had a median progression-free survival of 7.0 months and overall survival of more than 18 months. These analyses were repeated with CTC enumeration at the first follow-up visit. Similar trends of significantly diminished median progression-free survival as well as overall survival were again seen in those patients with 5 or more CTCs at their first follow-up visit. It should be also noted that the 10 patients who died before the first follow-up visit had extremely high counts of CTCs in their baseline blood sample (counts of 9, 11, 15, 24, 111, 126, 301, 1143, 4648, and 23,618 CTCs per 7.5 mL of blood,

respectively). Interestingly, the patients who had 5 or more CTCs at baseline but less than 5 at first follow-up visit had progression-free and overall survival rates that were not statistically different from those patients with less than 5 CTCs at both baseline and first follow-up visit. Likewise, the group of patients with less than 5 CTCs at baseline but 5 or more at first follow-up had progression-free and overall survival rates that were not statistically different from the group of patients who had 5 or more CTCs at both time points. The findings of this trial suggest that the level of CTCs before initiation of new therapy and the level of CTCs at follow-up visit can be valuable markers to predict progression-free survival and overall survival in metastatic breast cancer patients.

3.2 Assessment with CTCs Versus Traditional Imaging Studies

Soon after the above data were available, Budd et al. published their findings that assessment of CTCs in metastatic breast cancer patients can determine disease status earlier and more reliably than traditional imaging studies (Budd et al. 2006). This group of researchers carried out a prospective, double-blinded clinical trial at 20 centers throughout the USA to compare use of CTC enumeration with radiologic studies in 138 metastatic breast cancer patients. Prior to initiation of new systemic therapy, these patients had computed tomography and/or magnetic resonance imaging scans of chest and abdomen, a whole-body bone scan, and a baseline count of CTCs. Each patient's disease status was reassessed every 9 to 12 weeks with imaging studies and at monthly intervals with blood draws for enumeration of CTCs using CellSearch® System.

In this study, interreader and intrareader discrepancies were found to be much greater between the two radiologists reading the radiologic images, when compared to interreader and tube-to-tube variability for the CTC assay (Budd et al. 2006). Interreader variability was 15% with imaging, while only 1% with the CTC-based method. Interestingly, while there was no correlation between tumor sizes as measured by the radiologists and CTC levels, therapeutic response, as measured by radiologic images, was concordant with CTC levels in 76% of all cases. Both radiologic and CTC-based methodologies were used to predict survival. The median overall survival for patients with less than 5 CTCs at the first follow-up visit after initiation of therapy was 22.6 months, in contrast to 8.5 months for patients with 5 or more CTCs. Seventy percentage of patients with radiologic response to therapy, as defined by stable disease or regression of disease, had the median overall survival of 24.9 months, in contrast to 12.9 months for the patients with radiologic findings consistent with progression of disease. Further analysis revealed that within the group of "responders," as defined by the CTC counts, radiographic responders and non-responders did not have a significantly different prognosis. However, within the group of "responders" as defined via radiological studies, CTC responders and CTC non-responders had a significantly different prognosis (26.9 months vs. 15.3 months). The converse was also true. Within the group of "non-responders" determined by CTC counts, radiographic responders and

non-responders did not have a significantly different prognosis. In contrast, within the group of "non-responders" by radiology, CTC responders and non-responders had a significantly different prognosis (19.9 months vs. 6.4 months). This study suggests that CTC enumeration may be a more reproducible and more robust predictor of survival than traditional radiographic studies that have been used to assess the efficacy of breast cancer treatment. More reliable assessment of response to treatment may spare patients from the adverse effects and toxicities of systemic therapies that ultimately may have no significant benefits and can allow clinicians to better tailor the course of treatment and consider alternative therapies earlier.

3.3 CTC Counts to Predict Resistance to Therapy in Metastatic Breast Cancer Patients

The poor prognosis of patients with elevated levels of CTCs before or during anticancer treatment may indicate resistance to therapy. Therefore, SWOG Protocol S0500 was conducted to evaluate if early change in treatment plan in patients with persistently elevated CTC levels would change the outcome (Smerage et al. 2014). This randomized trial enrolled 595 female patients with histologically confirmed primary breast cancer and with clinical and/or radiographic evidence of metastatic disease, who did not receive any prior chemotherapy for metastatic disease. The choice of chemotherapy was at the discretion of the treating physician; only patients who were to be treated with a single agent were eligible for this study. Patients with CTC levels less than 5 per 7.5-mL blood at baseline (arm A) as detected by the CellSearch® System were continued on the chemotherapy initiated at their physicians' discretion until there was progression of disease. Those patients who had 5 or more CTCs at baseline had repeated CTC enumeration approximately 22 days after the first administration of chemotherapy. Those patients who had decreases in CTC counts to less than 5 CTCs (arm B) continued on to receive a second cycle of first-line chemotherapy. Patients who had persistently elevated CTC levels were randomized to the group who continued to the same first-line chemotherapy regimen (arm C1) or to the group who switched to an alternative regimen (arm C2). In congruence with the 2004 Cristofanilli study, 54% had 5 or more CTCs at baseline. Of those with elevated CTC levels at baseline, 57% no longer had elevated levels at first follow-up. Of the remaining 123 patients who had persistently elevated CTCs, 87% died and 99% experienced disease progression. Again, elevated CTC levels portended worse prognoses, as median overall survival for arms A, B, and C (arms C1 and C2 combined) was 35 months, 23 months, and 13 months, respectively. Regrettably, for those with persistently elevated CTCs, earlier transition to an alternative cytotoxic treatment did not change the poor outcome. The median overall survival observed in arms C1 and C2 was 10.7 months and 12.5 months, respectively; the median progression-free survival observed in arms C1 and C2 was 3.5 months and 4.6 months, respectively. This result suggests that patients with persistently increased CTCs despite cytotoxic therapy may represent a group whose tumors are resistant to not only one, but also several commonly used anticancer

agents. Therefore, continued therapy with other standard regimens likely will not provide substantial benefit. Thus, participation in trials of novel therapies may be a preferable alternative than exposure to the toxicities of second-, third-, or ever later and less desirable lines of chemotherapy.

4 Circulating Tumor Cells in Non-metastatic Breast Cancer

Metastatic cases only account for 5–8% of total newly diagnosed breast cancer cases (Jemal et al. 2010). Therefore, more recently, other research groups have studied clinical implications of CTCs in the non-metastatic breast cancer patient population.

4.1 CTCs as Micrometastasis Biomarkers in Early-Stage Breast Cancer

Dissemination of CTCs into the circulation has been detected in patients with small primary tumors (Nadal et al. 2013). Using the CellSearch® System, Krishnamurthy et al. detected CTCs in 31% of patients with T1 or T2 primary tumors (Krishnamurthy et al. 2010). Similar detection rates in early-stage breast cancer patients were seen in other studies as well (Bidard et al. 2010; Franken et al. 2012; van Dalum et al. 2015). In this patient population, the presence of CTCs had no correlation with traditionally accepted prognostic factors, such as tumor size, tumor histologic grade, hormone receptor status, HER2 status, and lymph node status (Krishnamurthy et al. 2010). The presence of CTCs in early-stage breast cancer patients suggests that advanced disease is not necessary for cancer cells to spread hematogenously and that these patients have dissemination of tumor cells that are undetected by standard imaging technologies.

4.2 Prognostic Values of CTCs in Non-metastatic Breast Cancer

At our institution, the University of Texas MD Anderson Cancer Center, CTC enumeration using the CellSearch® System was performed in 509 patients with non-metastatic breast cancer patients prior to surgical resection of the primary tumor (Hall et al. 2016). In this study, the presence of 1 or more CTCs per 7.5 mL of blood was used as the primary cutoff. One or more CTCs were found in 24% of patients, with 2 or more in 7.5%, and 3 or more in 5% (Hall et al. 2016). Among 509 patients, 166 patients (33%) had received neoadjuvant chemotherapy prior to CTC enumeration, and CTCs were found in 26% of those 166 patients. Similarly, 24% of chemotherapy-naïve patients had detectable CTCs in their blood sample. Again, there was no statistically significant correlation between the presence of CTCs and tumor size, tumor grade, hormonal receptor status, HER2 status, and

lymph node status. During the 48-month follow-up period, 51 patients (11%) relapsed after surgical resection of primary tumor, with distant metastasis in 46 patients. Sites of distant metastasis were not associated with the presence of CTCs. Of 124 patients with 1 or more CTCs, 19% relapsed. In contrast, only 7% of patients with no CTC prior to surgery relapsed. Both univariate and multivariate analyses demonstrated that the presence of CTC in non-metastatic breast cancer patients predicted decreased relapse-free survival. Furthermore, hazard ratios for relapse increased with increasing CTC counts. There were 31 deaths in this study. Thirteen of 125 patients with CTCs died, compared to 18 of 385 patients with no CTCs. Although overall survival was poorer in patients with 1 or more CTC, there was no statistical significance with multivariate analysis. However, patients with 2 or more CTCs per 7.5 mL of blood had statistically significant decrease in overall survival, compared to patients with no CTCs.

When the presence of CTCs was measured in a subset of patients who are chemotherapy-naïve at the time of surgery for their primary tumor, a similar trend was observed (Lucci et al. 2012). In this study, 24% of 302 patients had at least one CTC per 7.5 mL of blood. Having one or more CTC predicted significantly lower progression-free and overall survival, and hazard ratios for disease progression increased with the increasing number of CTCs. And as previously discussed, there was no association between CTC counts and axillary lymph node status. However, higher numbers of CTCs carried hazard ratios as prognostically powerful as lymph node metastasis.

The fact that the level of CTCs can predict worse prognosis in early-stage breast cancer patients without a significant association with lymph node status suggests that CTC counts may be able to detect micrometastatic hematogenous spread and identify additional patients at higher risk of treatment failure, independent of degree of lymphatic involvement. It should also be noted that not all patients with CTCs will relapse. Therefore, it is a marker for increased risk but not an absolute indicator of relapse. Considering previously established data that 20% of lymph node-negative patients also relapse (Harbeck and Thomssen 2011), routine CTC evaluation may identify more high-risk patients.

The largest known study in non-metastatic breast cancer patients is a pooled analysis by Janni et al. of 3173 stage I to III breast cancer patients from 5 academic breast cancer centers (Janni et al. 2016). In this study, the presence of CTCs was assessed at the time of primary diagnosis, using the CellSearch® System. One or more CTCs were detected in 20.2% of patients. Unlike the other smaller studies, there was a statistically significant association between the presence of CTCs with larger tumor size, increased lymph node involvement, and unfavorable histologic grade. In patients without CTCs, 45.8%, 44.9%, 5.8%, and 3.0% of patients had T1, T2, T3, and T4 tumors, respectively, in contrast to 38.1%, 49.1%, 8.6%, and 3.8% of patients with CTCs who had T1, T2, T3, and T4 tumors, respectively. Of patients without CTCs, 54.8% had nodal involvement versus 60.9% of patient with one or more CTCs. Histologic grading similarly demonstrated a weak trend for more aggressiveness in patients with CTCs that showed statistical significance in this large study. The presence of one CTC or more per 7.5 mL of blood was as an

independent prognostic factor for disease-free survival as well as distant disease-free survival, breast cancer-specific survival, and overall survival. Additional subgroup analysis was performed in this study, which showed an exception, that CTC presence was not a prognostic factor in very early-stage breast cancer patients with T1N0 tumors. Thus, in this specific group, the current standard of care will likely treat patients successfully, independent of CTC status and with low risk of relapse. Lack of prognostic significance was also found in patients with HER2-positive and hormone receptor-negative tumors. However, when all HER2-positive patients were analyzed together, there was a prognostic significance. This may be due to variable responsiveness to chemotherapy in HER2-positive tumors, depending on the hormone receptor status.

4.3 Implication of CTCs in Pathologic Complete Response and Prognosis

In a recent study that analyzed CTCs in 63 stage III inflammatory breast cancer patients after primary systemic therapy, the presence of CTCs was found to have no association with complete pathologic response to or the failure of chemotherapy (Hall et al. 2015). Despite no relationship between the presence of CTCs and the complete pathologic response rate, they both independently predicted shortened relapse-free survival. In this study, 25.8% of patients had pathologic complete response after completion of primary systemic therapy, and one or more CTCs were detected in 27% of patients after systemic therapy. There were 23 patients with relapse, and 12 patients died due to breast cancer-specific causes. Ten of 17 patients (58.8%) with detectable CTCs relapsed, in contrast to 13 of 46 (28.3%) patients without any detectable CTCs. Furthermore, relapse hazard ratios increased with the increase in the number of CTCs.

Pierga et al. also studied the relationship between CTCs and pathologic complete response in non-metastatic stage III inflammatory breast cancer patients who were enrolled in phase II multicenter trials, BEVERLY-1 and BEVERLY-2 (Pierga et al. 2017). Among 137 patients, 39% had detectable CTCs at baseline. After 4 cycles of systemic therapy, the CTC detection rate decreased to 9%, and the pathological complete response rate was 40%. Despite lack of correlation between CTC and pathological complete response rate, the presence of CTC at baseline and inability to achieve pathological complete response were again both independent prognostic factors for decreased disease-free and overall survival rates. Furthermore, in this group of patients with extremely aggressive disease, a subset of patients who had no detectable CTCs at baseline and had pathological complete response after neoadjuvant therapy had an exceptionally excellent outcome with 88% disease-free survival and 94% 3-year overall survival.

Historically, measuring pathological complete response rates has been used to assess tumor sensitivity to treatment and has correlated well with improved outcome (Gebreamlak et al. 2013). However, not all patients with pathological complete response remain disease-free with long-term follow-up. And, a recent

meta-regression analysis of 29 neoadjuvant trials did not support the use of pathological complete response as a surrogate marker to predict disease-free survival and overall survival (Berruti et al. 2014). CTC may be a useful marker to identify patients who remain at high risk for relapse, despite achieving pathological complete response, as exceptional response to systemic therapy within local and/or regional area cannot completely revert the risk of micrometastases represented by the CTCs. Therefore, baseline CTC enumeration should be considered as a stratification protocol for future breast cancer clinical trials. Of note, because of the low number of non-metastatic cases with multiple CTCs per 7.5 mL of blood, metastatic breast cancer patient studies typically used a cutoff level of 5 CTCs, while non-metastatic breast cancer studies used a cutoff of 1 CTC per 7.5 mL of blood.

5 Circulating Tumor Cells in Current Clinical Practice

5.1 Southwest Oncology Group Trial S0500

In SWOG Trial S0500, metastatic breast cancer patients with persistently elevated CTCs after 21 days of first-line chemotherapy were switched early to an alternative cytotoxic therapy. This early transition based on knowledge from repeat assessment with CTC enumeration did not translate into more positive outcomes. However, because this study did not measure response to therapy or quantify clinical benefit from individual chemotherapy agents, one cannot conclude that those patients with persistently elevated CTCs derived no benefit from those agents. Nonetheless, their data suggest that those who are clearly refractory to first-line chemotherapy are likely to be refractory to other commonly used chemotherapeutic agents. Therefore, instead of receiving cytotoxic agents that may not provide substantial benefits, those patients can move onto novel therapies, such as targeted therapies that are in early phase clinical trials before patients' performance statuses and clinical situations become too poor or severe to enroll in those trials.

5.2 American Society of Clinical Oncology (ASCO) Guidelines

With many studies that have been published and in progress on various breast tumor biomarker assays, ASCO has published evidence-based recommendations to guide clinicians on how to make clinical decisions based on biomarker findings. Currently, for early-stage non-metastatic breast cancer patients, ASCO states "the clinician should not use circulating tumor cells (CTCs) to guide decisions on adjuvant systemic therapy," as CTCs have repeatedly demonstrated prognostic value but not yet shown direct clinical utility (Harris et al. 2016). In regard to metastatic breast cancer patients, multiple studies have described CTCs as poor prognostic predictive markers. However, the only study that relied on CTCs as markers to guide therapy showed no difference in outcomes (Smerage et al. 2014).

Therefore, current ASCO guideline states that "in patients already receiving systemic therapy for metastatic breast cancer, decisions on changing to a new drug or regimen or discontinuing treatment should be based on the patient's goals for care and clinical evaluation and judgment of disease progression or response, given that there is no evidence at this time that changing therapy solely on the basis of circulating biomarker results improves health outcome, quality of life, or cost-effectiveness (Van Poznak et al. 2015)." Therefore, further studies to assess clinical utility are urgently needed before CTC enumeration can be used routinely to guide therapeutic decisions.

5.3 National Comprehensive Cancer Network (NCCN) Guidelines

As in ASCO guidelines, the clinical use of CTCs is not currently included in the NCCN guidelines for breast cancer disease assessment and monitoring. CTC counts have not yet shown a predictive value. The presence of CTC and CTC counts can provide valuable information on the likely outcome of breast cancer disease and identify high-risk patients who are more likely to relapse after standard treatment. However, we do not currently have any data to show that CTCs can provide information on relative sensitivity or resistance to the various standard treatments. That is, we cannot use CTCs to identify patients who will benefit from a given therapy. Therefore, as noted previously, while CTCs can be used to counsel patients on their overall prognosis, CTC presence and enumeration cannot yet be used for the purpose of optimizing therapy decisions.

5.4 DETECT Studies: Investigating Use of CTC Phenotypes for Guiding Clinical Decisions

Due to lack of evidence for the predictive value of CTCs in terms of treatment outcomes, and growing evidence for intratumor and temporal heterogeneity of breast cancer genomes, the DETECT studies are currently looking into the clinical utility of assessing CTC phenotypes to guide therapeutic decisions (Arkadius Polasik et al. 2016; Schramm et al. 2016). In a study of 254 metastatic breast cancer patients, Fehm et al. compared primary tumor and CTC HER2 statuses and revealed that 30% of patients classified as HER2-negative on tumor biopsy exhibited HER2-positive CTCs (Fehm et al. 2010). As one of the first interventional trials to validate clinical utility of CTCs, the DETECT III trial is comparing standard therapy alone versus addition of HER2-targeted therapy to standard therapy in those patients with initial HER2-negative metastatic breast cancer but HER2-positive CTCs (Schramm et al. 2016). This prospective, multicenter, randomized phase III clinical trial will assess the efficacy of lapatinib in patients with HER2-positive CTCs as well as the significance of CTCs as an early predictive marker for treatment response. Another clinical trial that is currently recruiting participants is the

DETECT IV trial, a prospective, multicenter, randomized phase II clinical trial that will study patients with HER2-negative metastatic breast cancer with persistently HER2-negative CTCs (Schramm et al. 2016). Here, hormone receptor-positive patients will receive everolimus and the clinician's choice of endocrine-based therapy. Those with triple-negative disease or with indication for chemotherapy will receive eribulin. The effects of treatment with everolimus will be assessed in relation to change in the PI3K/Akt/mTOR pathway in CTCs as well as ESR-1 mutation status in CTCs. DETECT V study was also initiated in 2015 for metastatic patients with triple-positive disease (Schramm et al. 2016). Here, patients are randomized to a dual HER2-directed therapy with pertuzumab and trastuzumab with either chemotherapy or endocrine therapy. Aim here is to assess genomic expression of CTCs to determine endocrine responsiveness score in order to predict likelihood of successful treatment. Together, the DETECT studies will evaluate the predictive role of CTCs to guide more personalized therapy for patients with metastatic breast cancer.

6 Tumor Heterogeneity: Circulating Tumor Cells and Circulating Tumor DNA

The discrepancy mentioned above between many patients' primary tumor and CTC HER2 status is just one example of the ubiquitous genetic heterogeneity of breast carcinomas, which poses major challenges in optimizing treatment regimens (Ellsworth et al. 2017). Heterogeneity exists among breast tumors from different patients, intratumor heterogeneity within a single tumor mass, heterogeneity between the primary tumor and subsequent metastatic lesions, as well as temporal heterogeneity over the course of tumor growth or in response to anticancer therapies (Ellsworth et al. 2015, 2017; Torres et al. 2007). Studies have shown great genomic variations between primary breast carcinomas and paired metastatic lesions (Ellsworth et al. 2017; Kuukasjarvi et al. 1997). And while metastases are largely responsible for breast cancer mortality, current risk stratification and treatment recommendations continue to rely on histological and molecular characteristics of the primary tumor (Ellsworth et al. 2017). We do not yet know if changing a course of treatment based on liquid biopsy biomarkers is a worthwhile endeavor when there is discordance between the primary tumor and CTCs. The ongoing DETECT studies discussed in brief above will be extremely informative in adjusting systemic chemotherapy and targeted therapy as our patients undergo treatment and tumors and their metastatic lesions evolve.

One potential reason that CTCs have yet to been found to predict therapeutic benefit is that molecular heterogeneity is also found among CTCs (Ellsworth et al. 2017). A single-cell analysis study has shown that there are different mutation patterns of PIK3CA gene among CTCs and DTCs (Deng et al. 2014). Another study that looked at 50 cancer-related genes using next-generation sequencing also found great heterogeneity between CTCs and the primary tumor, as well as among

CTCs from the same patient (De Luca et al. 2016). It is possible that the abundant heterogeneity between primary tumors, CTCs, and DTCs represents chances for refractoriness to therapy and/or disease relapse to occur. Therefore, these recent studies together suggest that a liquid biopsy method that can capture genomic signatures of all cancer cells may provide more informative and actionable genomic data.

Therefore, researchers have started to look at cell-free DNA (cfDNA) as another source of more comprehensive genomic information. As cells undergo apoptosis, necrosis, and macrophage phagocytosis, they release nucleic acid fragments called cfDNA into the bloodstream (Ellsworth et al. 2017). A subset of cfDNA, called circulating tumor DNA (ctDNA), is more abundant than CTCs and is more dynamic in that it is rapidly cleared from circulation within hours (Ellsworth et al. 2017). Furthermore, ctDNA in metastatic breast cancer patients has shown to accurately represent the mutational profile of individual CTCs (Shaw et al. 2017). Therefore, over the last several years, there has been great enthusiasm in studying the clinical utility of ctDNA, especially in analyzing cancer-specific mutations in cfDNA (Ellsworth et al. 2017; Liang et al. 2016; Canzoniero and Park 2016; Dawson et al. 2013; Murtaza et al. 2013). Thus far, ctDNA levels have been shown to correlate well with changes in tumor burden and appear promisingly as a potential tool for monitoring the progression of breast cancer (Liang et al. 2016; Dawson et al. 2013). Performing serial analysis of ctDNA in plasma samples over time has allowed the tracking of genomic evolution in response to therapy (Liang et al. 2016; Murtaza et al. 2013). At this time, ctDNA has not yet been validated as a marker for routine, direct use at the treatment level; however, there are many potential applications, including residual disease detection following treatment, noninvasive tumor genotyping, and early detection of relapse (Ellsworth et al. 2017).

7 Future Directions

7.1 Comprehensive CTC Detection

Studies included in this chapter used the CellSearch® System, the only FDA-approved method at this time. There is a major limitation to this method, as it only detects CTCs with the epithelial-specific marker, EpCAM. A concern with the positive selection process based on EpCAM and cytokeratin expression to isolate CTCs is that it may exclude CTCs that are undergoing epithelial–mesenchymal transition (EMT), as these cells have reduced level expression of epithelial markers such as EpCAM (Hall et al. 2016; Krawczyk et al. 2014; Bidard et al. 2016). EMT is a process by which epithelial carcinoma cells reprogram and attain cellular properties seen in mesenchymal cells, such as motility, invasiveness, and resistance to apoptosis (Krawczyk et al. 2014; Bidard et al. 2016; Dave et al. 2012). It is thought to be induced by the tumor microenvironment, ultimately leading a local spread of cancer cells, and has been associated with cancer progression and

increased stemness, or stem cell-like behavior, of tumor cells (Krawczyk et al. 2014; Bidard et al. 2016; Dave et al. 2012; Mego et al. 2010). Interestingly, increased EMT markers have been linked with aggressiveness of metastatic disease as well as intrinsic resistance to anticancer therapies (Hall et al. 2016; Dave et al. 2012; Mego et al. 2010), and mesenchymal CTCs were more frequently observed during the period of tumor progression (Krawczyk et al. 2014; Bidard et al. 2016). This means that the more aggressive CTC types that are undergoing phenotypic changes associated with EMT, understood as an essential process for cancer metastasis, are not detected by the CellSearch® System. Therefore, the technology that will capture a snapshot of all CTCs is necessary to fully elucidate the biology of CTCs and potentially bridge the gap between CTCs as cancer phenomena and CTCs as guides for clinical care.

7.2 Functional Characteristics of CTCs

Because endocrine therapy fails to produce response in half of hormone receptor-positive patients, Paoletti et al. have developed a CTC-endocrine therapy index (CTC-ETI) to predict resistance to therapy (Paoletti et al. 2015). In addition to CTC enumeration by the CellSearch® System, the CTC-ETI quantifies CTC heterogeneity by measuring the expression levels of 4 markers by immunofluorescent staining: estrogen receptor (ER), B-cell lymphoma 2 (BCL-2), human epidermal growth factor receptor 2 (HER2), and Ki67. This study showed significant CTC heterogeneity for each biomarker and led to the hypothesis that patients with high CTC-ETI scores (high heterogeneity) are more likely to be resistant to endocrine therapy. There are two clinical trials, COMETI P2 (NCT01701050) and SWOG S1222 (NCT02137837), evaluating the clinical validity of CTC-ETI (Paoletti et al. 2015). COMETI P2 has been completed with results pending. SWOG S1222 is ongoing.

7.3 Interventional Clinical Trials to Assess Clinical Application of CTCs

7.3.1 STIC CTC METABREAST Trial (NCT01710605)

In this trial, hormone receptor-positive metastatic breast cancer patients will be randomized between a standard arm where treatment will be decided by clinicians using standard criteria and a CTC-based arm where treatment will be decided by the CTC count. In the CTC arm, hormone therapy will be given as the first-line treatment if there are less than 5 CTCs per 7.5 mL of blood by the CellSearch® System, and chemotherapy will be given as the first-line treatment if there are 5 or more CTCs per 7.5 mL of blood. The main objective will be to evaluate for the non-inferiority of the CTC-based clinical decision making relative to care using the current standard criteria (Bidard et al. 2013).

7.3.2 CirCe01 (NCT01349842)

This trial tests the efficacy of early changes/switches in the course of chemotherapy for patients whose CTC counts did not decrease after the first cycle of chemotherapy, based on the assumption that CTCs may be markers for chemoresistance. This study's hypothesis is that CTC analysis will allow early discontinuation of inefficient yet toxic and costly therapies. Patients with a high CTC count before starting third-line chemotherapy will be randomized to a CTC-driven arm or a standard arm. In the CTC-driven arm, CTC counts will be repeated after each first cycle of new chemotherapeutic agents. Those patients with persistently high CTCs will be switched off that agent, while patients with a significant drop in CTC counts will continue on their treatment (Bidard et al. 2013).

7.3.3 Treat CTC

Currently, trastuzumab is administered as the standard of care in patients with HER2-amplified breast cancers. However, additional studies have suggested the benefit of trastuzumab treatment in patients without known HER2 amplification; this trial assesses if non-metastatic patients with non-amplified HER2 tumors but with one or more CTC, regardless of CTC HER2 status, can benefit from trastuzumab treatment (Bidard et al. 2013). Patients with one or more CTCs identified by the CellSearch® System will be randomized after completion of neoadjuvant chemotherapy and surgery to a trastuzumab treatment arm or an observation-only arm.

7.4 Clinical Trials for Targeted Therapy

There are other ongoing clinical trials to assess the clinical benefit of CTC characterization for personalized targeted therapy. In an ongoing, single-arm phase II clinical trial (NCT01975142), HER2-negative metastatic breast cancer patients are being screened for CTC HER2 amplification; if CTC HER amplification is present, they are treated with trastuzumab emtansine. In another phase II clinical trial (NCT03070002), the efficacy of denosumab is being tested in patients with ER- and/or PR-positive, HER2-negative metastatic breast cancer patients with bone metastases; CTC enumeration is utilized as an indicator for response to therapy.

8 Conclusions

In current clinical practice, a single tumor biopsy remains the main diagnostic tool to guide the course of therapy. However, it is now clear that this invasive method only shows a minority of tumor cells among the myriad of heterogeneous tumor cell populations and will likely underestimate mutational burden. Because of the invasive nature of tissue biopsies as well as the cost, breast cancer patients rarely undergo tissue biopsy of metastatic lesion. Therefore, liquid biopsies of CTCs

and/or ctDNA show great promise as means for serial monitoring and surveys of breast cancer heterogeneity as a tumor responds to treatments or progresses. Optimization of personalized medicine to effectively treat breast cancer patients is urgently needed. To bring these liquid biopsy methods into the realm of routine clinical decision making and monitoring, large clinical trials in both early- and late-stage breast cancer patients are necessary to determine if the alteration of targeted therapies based on comprehensive genomic characterization and serial monitoring will provide meaningful improvements in the clinical outcomes of breast cancer patients.

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Clinical Applications of Circulating Tumor Cells in Breast Cancer

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1 Introduction

The development of metastatic disease accounts for the vast majority of cancer-related deaths in solid tumor malignancies. Distant metastases primarily develop as a result of tumor cell dissemination through the circulatory system (Chaffer and Weinberg 2011). Over the past several decades, several technologies have been developed to isolate, enumerate, phenotype, and genotype circulating tumor cells (CTC) from the peripheral blood. While successful isolation of CTC has been reported in numerous solid tumor malignancies (Scher et al. 2011; Coget et al. 2012; Poruk et al. 2016), breast cancer has served as one of the most widely studied for the clinical application of CTC. In this chapter, we will review the studies that have established the analytic validity, clinical validity, and clinical utility of CTC enumeration as a prognostic biomarker in breast cancer. In addition, we will review ongoing clinical trials and research efforts that have the potential to establish the clinical utility of CTC enumeration and phenotyping and genotyping as a predictive biomarker to select therapies that are most likely to benefit patients.

1.1 Why Are CTC of Interest Clinical?

Of the many steps required for development of metastases, circulation of malignant cells to distant sites is the primary mechanism of the metastatic process in solid tumor malignancies. Isolation and phenotyping and genotyping of malignant cells in the circulation have the ability to enhance our understanding of mechanisms of

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resistance and predictors of response to antineoplastic therapies, thus leading to personalized oncologic care with greater precision. Historically, sampling of tumor cells to identify these prognostic and predictive factors has occurred through a tissue biopsy. For example, all patients with breast cancer undergo a biopsy to determine if the tumor cells express estrogen receptor (ER) since ER positivity is predictive of benefit from hormonally based therapies (Early Breast Cancer Trialists' Collaborative et al. 2011). However, these biopsies are fraught with several problems: (1) They are invasive and therefore inconvenient and relatively high risk, and (2) they are expensive, often requiring interventional radiology and pathology input (Table 1). These two issues hamper easy collection of metastatic disease and almost prohibit serial sampling of metastases during a patient's clinical course. Furthermore, metastatic sites may have dramatically different mutational profiles, implying that tumor heterogeneity may not be accurately reflected by analysis of a single metastatic lesion (Gerlinger et al. 2012).

Taken together, these issues make characterization of CTC from the peripheral blood an attractive concept. Perhaps isolating, phenotyping and genotyping a variety of malignant cells within the circulation can better overcome the problem of sampling bias that results from testing a single metastatic site, and permit monitoring changes simply and easily with serial blood draws (Table 1). These considerations have led to the concept that a blood draw may serve as a "liquid biopsy" (Alix-Panabieres and Pantel 2013). A liquid biopsy could measure several different components of human blood: soluble protein, nucleic acids, metabolites or whole cells, specifically CTC. For example, circulating tumor-associated proteins such as carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), CA 125, CA 19-9, and assays for MUC 1 protein (CA 15-3, CA 27.19) have all been incorporated into routine monitoring of patients with colorectal, prostate, ovarian, pancreas, and breast cancers, respectively.

More recently, polymerase chain reaction (PCR)-based methods to isolate and sequence fragments of cell-free circulating tumor DNA (ctDNA), which are presumed to originate from dying malignant cells, are also being employed to characterize tumors. Sequencing ctDNA has numerous potential clinical applications which include: (1) identifying molecular mechanisms of resistance to treatment and (2) assessing response to therapy (Nygaard et al. 2013). For example, mutations in the gene encoding ER (*ESR1*) have been identified as a mechanism of resistance to endocrine therapy (ET) in patients with metastatic ER-positive breast cancer (Robinson et al. 2013). These mutations were originally discovered through next-generation sequencing (NGS) analyses of metastatic tumor tissue. More recently, they have also been identified in ctDNA (Chu et al. 2016; Schiavon et al. 2015). Importantly, *ESR1* mutations identified in ctDNA are not always identified in tumor tissue from the same patient, implying that assessment of a circulating biomarker may provide a more scrupulous method of characterizing a malignancy.

However, none of these soluble circulating tumor biomarkers permit cellular phenotyping, which can only be performed through capture of a CTC. Since CTC is also a circulating biomarker with similar potential clinical applications, it is

Table 1 A Comparison of tissue sampling, circulating tumor cell, and cell-free circulating tumor DNA in patients with solid tumor malignancies

	Metastatic biopsy	Circulating tumor cell (CTC)	Cell-free tumor DNA (ctDNA)
Logistical/practical considerations	Invasive, more difficult to obtain	Easy to obtain	Easy to obtain
	Expensive (interventional radiology, pathology)	Inexpensive (blood draw)	Inexpensive (blood draw)
	Serial testing less feasible	Easy serial testing	Easy serial testing
Pre-analytical considerations	More difficult to control (sample processing, such as time to fixation, may cause artifacts)	Easier to control: pre-specified fixative, anticoagulant in collection tube	Easier to control (pre-specified fixative, anticoagulant in collection tube)
Sensitivity	Abundance of cells (10^6–10^8 cells per biopsy)	Low cell number ($\sim$1–1000 cells per 7.5-mL whole blood)	Low nucleic acid content (10^2–10^4 ctDNA copies/mL whole blood)
Phenotypic assays	Immunohistochemistry (ER, PR, HER2/neu, PD-L1)	Immunohistochemistry (ER, PR, HER2/neu, PD-L1)	N/A
Genomic assays	Comprehensive next-generation sequencing (NGS) feasible (whole genome, exome, transcriptome, copy number analyses)	NGS feasible for candidate genes (n = 10–100) depending on volume of CTC/nucleic acid content	NGS feasible for candidate genes (n = 1–10) depending on volume of nucleic acid content
Biologic considerations	Only represents one tumor site	May represent more comprehensive assessment of tumor	May represent more comprehensive assessment of tumor
	Represents biology of tissue-based cancer at that site	May not represent biology of "tissue-based" cancer	Unknown
	Represents "live" cancer cells	Represents "live" cancer cells	Represents apoptotic cells or secreted exosomes

important to understand how these biomarkers differ (Table 1). Most notably, information obtained through analysis of an intact circulating malignant cell may be reflective of a different component of the tumor than that which is obtained through sequencing free nucleic acid from an apoptotic malignant cell. It is for this reason that these circulating biomarkers may be utilized clinically in a complementary fashion, providing a more comprehensive assessment of the malignant process.

1.2 Establishing CTC as a Tumor Biomarker with Analytical Validity, Clinical Validity, and Clinical Utility

Prior to routine use of a tumor biomarker to direct patient care, the assay must undergo rigorous scientific testing in both the preclinical and the clinical settings. The Evaluation of Genomic Applications in Practice and Prevention (EGAPP) established three evidence-based principles which should be met prior to use of a tumor biomarker in clinical practice (Teutsch et al. 2009). These principles include: (1) *analytic validity* (demonstrating the accuracy, reproducibility, and reliability of the assay), (2) *clinical validity* (demonstrating that the assay can divide one population into two distinct groups that have different clinical outcomes), and (3) *clinical utility* (demonstrating that utilizing the assay improves patient outcomes). The American Society of Clinical Oncology (ASCO) and National Comprehensive Cancer Network (NCCN) guidelines currently state that CTC does not have clinical utility in screening, risk assessment, differential diagnosis, or prediction of benefit from therapy in patients with breast cancer (Van Poznak et al. 2015). There are, however, robust data demonstrating the clinical validity of CTC as a prognostic biomarker. In this chapter, we will examine these studies in depth and review ongoing clinical trials aiming to establish the clinical utility of CTC as a predictive biomarker for selection of therapy.

1.3 Strategies to Capture and Enumerate CTC

Many different technologies have been developed to isolate CTC from whole blood. The techniques are divided into two main categories: (1) positive selection, whereby CTC is separated from normal hematopoietic constituents on the basis of expression of some type of distinguishing protein, such as epithelial cell adhesion molecule (EpCAM), and (2) negative selection, whereby CTC is isolated following techniques such as RBC lysis, depletion of CD45 positive cells, or isolation on the basis of cell size. More than 50 different platforms for isolation of CTC have been reported in the literature (Paoletti et al. 2012; Paoletti and Hayes 2016). However, CellSearch® (Janssen Diagnostics, LLC) is the most well-studied technology and the only with approval by the United States Food and Drug Administration (FDA).

2 CTC as a Prognostic Biomarker

2.1 Early Breast Cancer

The incidence of CTC in early breast cancer is lower than in metastatic disease. However, several studies have reported detection of CTC in stages I, II, and III breast cancer using RT-PCR for cytokeratin (CK) or whole-cell enumeration via CellSearch®. Using RT-PCR, approximately 40% of patients with stage I or II

breast cancer have detectable CTC, and this has been associated with worse prognosis (Xenidis et al. 2009; Ignatiadis et al. 2007). Similarly, in studies utilizing the CellSearch® assay, CTC is detected in 5–24% of patients, which again was associated with slightly worse prognosis than those patients who did not have elevated CTC (Lucci et al. 2012; Rack et al. 2014). A meta-analysis conducted by Zhang and colleagues also demonstrated that the presence of CTC in early breast cancer was associated with shorter disease-free survival (DFS) and overall survival (OS) (Zhang et al. 2012). A recent pooled analysis has also demonstrated that the presence of CTC at time of diagnosis of breast cancer is an independent predictor of poor DFS, OS, breast cancer-specific, and distant disease-free survival (DDFS) (Fig. 1) (Janni et al. 2016). Further, the presence of CTC in the neoadjuvant treatment setting has also been correlated with poorer prognosis (Bidard et al. 2010). Recently, Bidard and colleagues reported that elevated CTC counts prior to beginning neoadjuvant chemotherapy are predictive of OS, DDFS, and locoregional recurrence-free survival (Bidard et al. 2016).

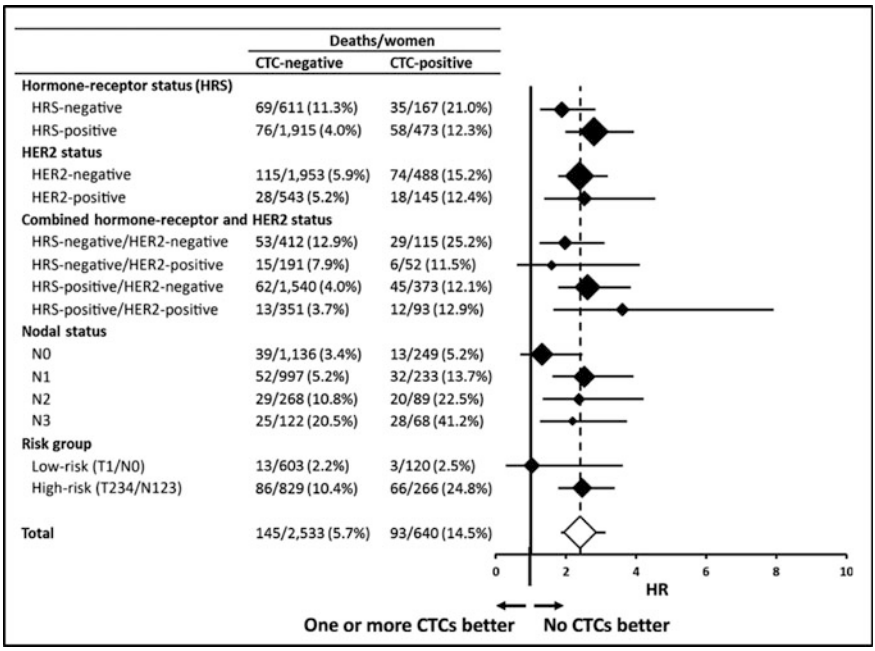

Fig. 1 Forest plot of overall survival in various breast cancer subgroups with and without CTC at time of breast cancer diagnosis. Black diamonds denote hazard ratios (HRs) for CTC positive versus CTC negative in subgroup analyses, and white diamond denotes overall HR for pooled analysis (vertical dashed line), which includes 3173 patients. The size of each diamond is proportional to the sample size in each group. Horizontal lines indicate 95% confidence intervals. Reprinted with permission from Janni et al. (Janni et al. 2016)

Taken together, these studies have demonstrated that CTC has established clinical validity in early-stage breast cancer as a prognostic biomarker. However, their clinical utility in this setting has not been established. No studies to date have directed adjuvant systemic therapy administration on the basis of CTC. Further studies are needed to further define the role of CTC in the early breast cancer setting.

2.2 Metastatic Breast Cancer

Numerous studies have demonstrated that enumeration of CTC is a strong predictor of progression-free survival (PFS) and OS in patients with MBC (Zhang et al. 2012; Cristofanilli et al. 2004). In a seminal study utilizing the CellSearch® (Janssen Diagnostics, LLC) assay, 177 patients initiating a new line of therapy for treatment of metastatic disease underwent CTC enumeration prior to treatment and at first follow-up. Using a training cohort of 102 patient samples and subsequently a validation cohort of 75 samples, a CTC cutoff of ≥ 5 CTC per 7.5 mL of whole blood (WB) was established, identifying a population of patients with a statistically significant shorter median PFS (2.7 vs. 7.0 months) and OS (10.1 versus >18 months). In this same dataset, the prognostic value of CTC was also observed via serial measurements in patients with MBC on treatment. Patients had CTCs repeated 4 times over a 20-week period. Over time, patients who continued to have low CTC had a median OS >18.5 months. For patients in whom CTC became elevated at any time point, the median OS was 4.1 months. This leads to the conclusion that the detection of elevated CTC at any time during therapy is an indicator of subsequent rapid disease progression (Hayes et al. 2006). These data also suggest that CTC reduction denotes a "CTC response" early in the course of a new therapeutic regimen. In 2004, the FDA cleared the CellSearch® System for monitoring patients with MBC. These associations hold true in all breast cancer subtypes, including hormone receptor (HR)-positive, HER2-positive, and triple-negative disease.

The poor prognostic implication of elevated CTC at baseline and in follow-up after systemic treatment for MBC has been confirmed in numerous subsequent studies (Bidard et al. 2010; Liu et al. 2009). A pooled analysis reported by Bidard et al. that included data from 1944 patients from over 20 studies found that prediction of OS was significantly improved by the addition of baseline CTC to standard clinicopathologic models in the metastatic setting (Bidard et al. 2014). In addition, this study found that the addition of circulating tumor marker (CEA and CA 15-3) levels to this model did not add any additional prognostic value.

The ability to estimate prognosis is a crucial aspect of clinical assessment, particularly as patients and physicians are weighing potential risks and benefits of a treatment. In addition, routine monitoring of CTC in the metastatic setting could allow for the detection of resistance to treatment earlier than may be possible using standard radiographic methods, potentially shortening the time that patients are exposed to an ineffective therapy. Giordano and colleagues have reported a prognostic nomogram incorporating baseline CTC levels as well as a number of other

clinical factors (age, disease subtype, presence of visceral metastases, and performance status) to estimate probability of OS at 1, 2, and 5 years in patients with MBC starting first-line chemotherapy (Giordano et al. 2013). While this study certainly substantiates the prognostic importance of CTC, it does not establish the clinical utility of the assay.

However, monitoring serial CTC levels to determine an early CTC response might be of value in patient care. Patients who fail to clear CTC early in the course of therapy may be fundamentally resistant to that treatment (Cristofanilli et al. 2004). This observation led to the hypothesis that using CTC data to direct a change in therapy in this circumstance may improve patient outcomes, and this was the premise of the first prospective, randomized clinical trial conducted by SWOG that aimed to establish the clinical utility of CTC (Smerage et al. 2014). In this study, women with MBC starting first-line chemotherapy had CTC levels measured at baseline. Approximately, 50% of the patients screened did not have elevated CTC at baseline. These patients, designated Group A, were followed without any further CTC measurements.

The patients who did have elevated CTC at baseline (≥ 5 CTC per 7.5 mL WB) repeated CTC measurement following one cycle of chemotherapy. Approximately, 60% of these patients had a decline in CTC to <5 CTC per 7.5 mL WB, presumably indicating a response to therapy. This group of patients, designated Group B, remained on the same chemotherapy until evidence of disease progression or death. The remaining patients who continued to have elevated CTC following one cycle of chemotherapy ($n = 123$), designated Group C, were randomly assigned to remain on the same chemotherapy regimen (Group C1) or switch to an alternative chemotherapy of their oncologist's choice (Group C2). The primary endpoint of S0500 was OS.

Unfortunately, there was no difference observed in outcomes (either PFS or OS) between groups C1 and C2 (Fig. 2). Since these patients went on to receive many other chemotherapy treatments, the short median OS of this group indicates that patients who have persistently elevated CTC following one cycle of chemotherapy are likely to have disease that is fundamentally resistant to cytotoxic chemotherapy. Interestingly, the patients in arms C1 and C2 had primary breast cancers of varying subtypes: 89 patients with HR-positive disease, 27 patients with triple-negative disease, and seven patients with HER2-positive disease. Regardless of the difference in intrinsic subtypes, these patients have an incredibly poor prognosis (median OS of 10.7 months observed in arm C1 and 12.5 months in arm C2) suggesting that they require better treatment options. It is reasonable to consider these patients for early enrollment in clinical trials utilizing novel agents.

In summary, CTC measured at baseline prior to the start of a new therapy, at least when measured by CellSearch®, has established clinical validity as a prognostic biomarker. However, it is unclear if they have clinical utility in this setting. Currently, there is no evidence that switching drugs or changing therapeutic intensity (i.e., using combination chemotherapy regimens as opposed to single-agent therapy) will improve outcomes for patients with elevated CTC.

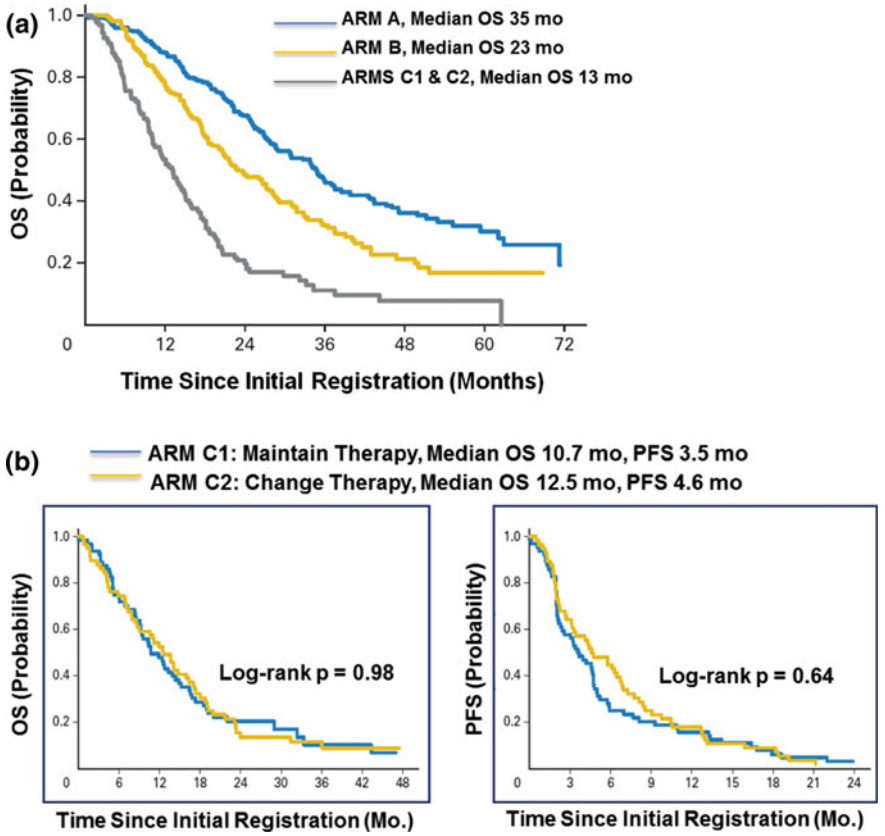

Fig. 2 **a** Overall survival of arms A, B, C1, and C2 in SWOG S0500 Study. Patients in Arm A had no detectable CTC at baseline prior to starting first-line chemotherapy for treatment of MBC. Patients in Arm B had detectable CTC at baseline, but cleared CTC following 1 cycle of chemotherapy. Patients in arms C1 and C2 had elevated CTC at baseline and did not clear CTC following 1 cycle of chemotherapy. Patients in Arm C1 continued the chemotherapy they were receiving previously, while those patients in Arm C2 received a different chemotherapy agent for cycle 2 of therapy. **b** Switching to an alternative chemotherapy agent did not improve progression-free or overall survival in patients with persistently elevated CTC following 1 cycle of chemotherapy, suggesting that these patients are fundamentally chemotherapy-resistant. Reprinted with permission from Smerage et al. (Smerage et al. 2014)

3 Investigational Efforts to Establish the Clinical Utility of CTC in MBC

3.1 CTC Enumeration to Direct Therapy

The previous discussions clearly demonstrate that CTC at baseline or follow-up has clinical validity in MBC, but as of yet no studies have demonstrated consensus

clinical utility. In this regard, there are several ongoing randomized clinical trials in Europe utilizing the CellSearch assay that aim to establish the clinical utility of CTCs to direct therapy. For example, the *STIC CTC* study is enrolling patients with ER-positive, HER2-negative MBC prior to first-line treatment in the metastatic setting and randomizing patients between standard therapy and baseline "CTC-directed" therapy. For patients randomized to the CTC-directed therapy arm, they are classified as low risk or high risk based on baseline CTC measurement. For those patients that have low levels of CTC at baseline (low risk), they are prescribed ET as first-line treatment. In contrast, those with high CTC levels at enrollment (high risk) are prescribed cytotoxic chemotherapy upfront. Similarly, the ongoing *CirCe01* study randomizes patients starting third-line chemotherapy between a standard therapy arm and a first follow-up CTC-directed therapy arm. For those patients on the CTC-directed therapy arm, CTC is obtained following the first cycle of cytotoxic therapy in the metastatic setting. Those patients with persistently elevated CTC are then immediately switched to an alternative treatment, even in the absence of clinical signs of progression. This same assessment then occurs after starting each subsequent therapy. One way in which this study differs from SWOG S0500 is that CTC is used to direct all subsequent lines of therapy (third, fourth, fifth, and subsequent lines) in the metastatic setting as opposed to just the first line of treatment.

3.2 Phenotyping and Genotyping CTCs to Predict Treatment Efficacy

The practice of precision medicine, or employing a therapeutic strategy based on a validated biomarker predictive of benefit from a particular therapy, has been utilized in the treatment of breast cancer since the discovery that patients with ER-positive disease benefit from anti-estrogen therapy while those with ER-negative disease do not (Early Breast Cancer Trialists' Collaborative et al. 2011). Similarly, it has been well established that patients with HER2-/neu-amplified breast cancer benefit from HER2-directed therapy (Moja et al. 2012). For these reasons, primary tumor tissue is routinely evaluated for these markers in all patients with breast cancer. However, there are cases of discordance in receptor status between primary and metastatic sites, indicating the need for biopsy of metastases to ensure that appropriate therapy is being administered (Lindstrom et al. 2012). Since a metastatic site may be difficult to biopsy, repeated biopsies at each point of documented progression are not feasible, and biopsy of a single metastatic site may not be reflective of the composition of the entire tumor burden, a liquid biopsy may eventually serve as a safer, less costly, more informative means of obtaining a tumor sample in patients with metastatic disease (Alix-Panabieres and Pantel 2013; Mathew et al. 2015).

Several groups have successfully identified important biomarkers in the management of breast cancer patients in CTC, including ER (Fehm et al. 2009), HER2 (Riethdorf et al. 2010; Meng et al. 2004), Ki-67 (Paoletti et al. 2015), BCL2 (Paoletti et al. 2015; Smerage et al. 2013), apoptosis (M-30) (Smerage et al. 2013),

IGFR1 (de Bono et al. 2007), EGFR (Payne et al. 2009), PI3K (Kallergi et al. 2007), gamma H2AX (Wang et al. 2010), PD-L1 (Mazel et al. 2015), and others. Paoletti and colleagues have developed a CTC-endocrine therapy index (CTC-ETI), evaluating relative expression of ER and BCL2 (both of which predict sensitivity to ET) and HER2 and Ki-67 (which predict resistance to ET) (Paoletti et al. 2015). Using the CellSearch® System, the CTC-ETI has been demonstrated to have high analytic validity. In this pilot study, CTC-ETI varied widely among 50 patients with ER-positive MBC, potentially identifying an ET refractory population who may benefit from chemotherapy earlier in their clinical course. A prospective clinical trial to address this question has completed accrual in North America (COMETI trial, ClinicalTrials.gov Identifier: NCT01701050), and initial results have been reported at the SABCS 2016 (Paoletti et al. 2016).

Similar to the concept of using ER status of CTC to direct therapy, several investigators have pursued use of HER2 status of CTC to select patients for HER2-directed treatments. In a phase II study conducted by Pestrin and colleagues, patients with HER2-positive CTC and HER2-negative primary tumors were selected for treatment with the oral tyrosine kinase inhibitor lapatinib (Pestrin et al. 2012). Unfortunately, no responses to lapatinib were observed among the patients treated in this manner. There are currently two larger ongoing studies aimed to determine the efficacy of HER2-directed therapy in patients with HER2-positive CTC and HER2-negative primary tumors (DETECT III, Clinicaltrials.gov Identifier: NCT01619111 and CirCEX1; Clinicaltrials.gov Identifier: NCT01975142). In the *DETECT III* study, patients with metastatic HER2-negative breast cancer starting first–third-line therapy have CTC assessed for HER2 expression at enrollment. Those who have HER2 expression found on CTC are then randomly assigned to receive or not receive lapatinib. The goal of this study is to determine whether or not HER2 expression on CTC may predict response to anti-HER2 therapy. The results of these randomized studies will provide valuable information regarding whether CTC number or marker expression may be of benefit in directing therapy in the metastatic setting.

Recently, immune checkpoint blockade with antibodies that target cytotoxic T lymphocyte antigen (CTLA-4) and programmed cell death protein 1 (PD-1/PD-L1) pathways have improved outcomes in a diverse group of malignancies (Postow et al. 2015). Presently, there are limited data available regarding the efficacy of immune checkpoint inhibitors in patients with breast cancer. However, preliminary data from ongoing clinical trials suggest some activity of these agents, with response rates ranging from 12 to 18% in patients with heavily pre-treated disease of varying histologic subtypes (Nanda et al. 2016; Rugo 2015). Studies in other cancer types have suggested that patients whose tumors stain positive for PD-L1 by immunohistochemistry have an increased likelihood of response to PD-1/PD-L1 inhibitors (Weber et al. 2013). In breast cancer, PD-L1 expression in tumor tissues varies considerably across disease subtypes (Ghebeh et al. 2006). Recently, Mazel and colleagues have demonstrated that PD-L1 is frequently expressed on the surface of CTC in patients with MBC (Mazel et al. 2015). This finding suggests that CTC may be utilized to determine PD-L1 positivity of a tumor, but additional

studies are needed to determine if CTC PD-L1 status correlates with findings in tissue and whether it predicts response to checkpoint inhibitor therapy.

There is also great interest in the potential utility of genotyping CTC, identifying somatic alterations that may provide insights into therapeutic targets or drug resistance. There are now multiple reports of successful detection of important tumor somatic mutations in circulating cell-free DNA and CTC from patients with breast cancer as well as other solid tumor malignancies (Dawson et al. 2013). It is currently unknown whether or not mutations identified in CTC will be similar to or different from those found in ctDNA. Of note, ctDNA may arise from lysed cells, raising concern that it may not be reflective of the genomic landscape of the viable tumor (Table 1). Genetic analyses of CTC, however, are limited by the fact that they must be purified from contaminating leukocytes.

Since most CTC capture platforms enrich, but do not purify CTC from other cellular components, technologies to accomplish this goal, such as dielectrophoretic Array (DEPArray™) System (Menarini Silicon Biosystems, Italy), have been developed. After completely purifying CTC, the DNA derived from either single or pooled CTC can be amplified and analyzed for genomic profiling (De Luca et al. 2016). In one such study, a patient with CTC was found to have three different *PIK3CA* mutation variants on individual CTC, underscoring the heterogeneity that may be uncovered through genomic analysis of CTC. While the clinical utility of genomic profiling of CTC has certainly not been established, this is an exciting area of ongoing investigation, particularly with regard to comparing results obtained from sequencing metastatic tissue and ctDNA.

4 Conclusion

In summary, CTC has been analytically and clinically validated as a prognostic biomarker in patients with early MBC. The clinical utility of CTC as a predictive biomarker to direct patient therapy has not yet been established. However, several clinical trials are ongoing and in development which may establish this paradigm. Given that patients with persistently elevated CTC following one cycle of first-line chemotherapy in the MBC setting have an incredibly poor prognosis and are fundamentally chemotherapy-refractory, and CTC enumeration in this context may be utilized in clinical trials as a tool to identify patients that may benefit from receiving novel therapeutic agents. Since these data were generated in patients with all histologic subtypes of breast cancer, this strategy may be useful to identify patients with traditionally “better prognosis” MBC, such as HR-positive disease, that are unlikely to benefit from standard therapies. In conclusion, utilization of CTC to serve as a liquid biopsy, perhaps in conjunction with other circulating biomarkers such as ctDNA, is an attractive concept in the era of precision medicine in oncology allowing for a noninvasive, relatively inexpensive, serial sampling of a tumor and a better assessment of tumor heterogeneity which could be utilized to better direct patient therapy.

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Circulating Tumor DNA (ctDNA)

Pathophysiology of ctDNA Release into the Circulation and Its Characteristics: What Is Important for Clinical Applications

Nickolas Papadopoulos

1 Introduction

cfDNA in human circulation was first reported in 1948 (Mandel and Metais 1948). ctDNA was postulated to exist as a component of cfDNA when elevation of cfDNA levels was observed in cancer patients (Leon et al. 1977). The concept of a liquid biopsy has percolated in the minds of clinicians and cancer biologists ever since these seminal findings and the discovery that cancer is a genetic disease. Gene mutations suddenly had potential as biomarkers unique to cancer tissue for the detection and treatment of the disease. It was not until at least 40 years later that it was reported that a fraction of cfDNA present in the plasma of cancer patients derives from cancer cells (Stroun et al. 1989), shortly followed by the first successful detection of mutations in body fluids performed in urine for the detection of *TP53* mutations from bladder cancer patients (Sidransky et al. 1991). Technological advances also contributed to the boom in the use of genomic analysis in liquid biopsy applications. Digital PCR (Vogelstein and Kinzler 1999) enabled accurate detection and quantification of rare ctDNA fragments. Many subsequent technological advances that preserved the digital analysis concept further contributed to the development of methods for the detection of cfDNA and ctDNA that progressed from utilizing single genes, to gene panels, to whole-exome sequencing (WES) and whole-genome sequencing (WGS) (Dressman et al. 2003; Diehl et al. 2005; Kinde et al. 2011; Forshew et al. 2012; Leary et al. 2012; Murtaza et al. 2013; Newman et al. 2014; Lanman et al. 2015). Perhaps justifiably, emphasis was placed on clinical applications, while the origins and the characteristics of cfDNA were studied sporadically. There are a number of reviews that cover what is known about

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Recent Results in Cancer Research 215,
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the nature, sources, and causes of cfDNA and ctDNA, and they also outline controversies and disagreements in the field (Schwarzenbach et al. 2011; Thierry et al. 2016; Wan et al. 2017; Aucamp et al. 2018). There remains, however, a lack of a complete understanding of the mechanisms that result in the presence of tumor DNA in the circulation, its potential fluctuations, and the kinetics of its clearance.

We still do not totally understand the relationship between the presence of ctDNA or the amount and the characteristics of the tumor. This deficiency in our knowledge is compounded by the considerable differences in DNA preparation and analytic techniques used in published studies. With such variability in techniques, it is difficult to directly compare the amounts of ctDNA among individuals with different tumor types (Diaz et al. 2012; Diehl et al. 2008; Forshew et al. 2012; Kuang et al. 2009; Taniguchi et al. 2011; Chang et al. 2002). Comparisons of studies are also challenging because of differences in the types of data that are reported. For example, real-time polymerase chain reaction (PCR) results are difficult to compare with the fraction of mutant template molecules assessed, or results based on the analysis of serum with those based on plasma. The very nature of ctDNA and its pathophysiology also presents challenges that need to be overcome for clinical assays to be effective. Some of them are technical, but some of them are biological.

The goal of this chapter is therefore to look at what we know now and what we need to know about ctDNA with the perspective of what is relevant to the development of clinical tests utilizing ctDNA as a cancer biomarker.

2 Biomarkers of ctDNA: How to Discriminate ctDNA from cfDNA

In the circulation of healthy individuals, the majority of the cfDNA is released from hematopoietic cells (Anker et al. 1975; Lui et al. 2002; Sun et al. 2015; Lehmann-Werman et al. 2016). cfDNA has been observed in both physiological and pathological conditions, including exercise, trauma, stroke, myocardial infarction, sepsis, diabetes, lupus, among others (Breitbach et al. 2012; Campello et al. 2007; Antonatos et al. 2006; Dwivedi et al. 2012).

We now know that ctDNA is a small fraction of cfDNA making its detection challenging. How can ctDNA be discriminated from cfDNA? Somatic mutations (point mutations, indels, rearrangements), copy number variation, aneuploidy, and methylation have all been used to identify ctDNA using a number of methodologies (Chan et al. 2013a, b; Leary et al. 2010; Murtaza et al. 2013; Leary et al. 2012; Douville et al. 2018). The sensitivity and specificity of each of them in detecting ctDNA in the presence of the more abundant cfDNA vary depending on the method used and the clinical application. Somatic mutations provide a qualitative marker that unequivocally distinguishes ctDNA from cfDNA, while aneuploidy and copy number variation provide a quantitative marker. Somatic mutations are present in one copy per cell, and their presence in circulation can be limited, especially in

patients with early-stage disease or micrometastasis. Furthermore, there are technical artifacts, such as PCR-based errors, that create background mutations increasing the noise. Rearrangements and indels have the advantage that they can be detected among millions of wild-type sequences, as supposed to thousands for point mutations, because technical errors do not generate specific rearrangements. Therefore, the signal-to-noise ratio is much higher than for point mutations (Leary et al. 2010; Bettegowda et al. 2014). The drawbacks for translocations are that they are rare in solid tumors and often are specific to individual cases requiring specialized tests. Methylation patterns in ctDNA have been used not only for the detection of cancers, but also for the detection of the tissue of origin of the cancer (Sun et al. 2015; Hao et al. 2017). A major drawback, at least for applications that require exquisite sensitivity, is that there are damage and loss of molecules during the preparation of the ctDNA template before sequencing which can limit the sensitivity.

Measuring quantitative changes in the genome provide another way of discriminating ctDNA from cfDNA. Amplified regions in the genome of tumor cells should be relatively enriched in the ctDNA, while deleted regions should be underrepresented. Because of the lack of a "beacon," like a mutation, quantitative changes usually require more events (which translates into more molecules) in order to increase confidence in the result, which in turn could limit sensitivity in certain clinical applications (Lo et al. 2010; Jiang et al. 2015). A mutation in an amplified region in the tumor genome would be a more ideal marker than either the mutation or the amplification alone. Aneuploidy is itself a tantalizing biomarker to evaluate in ctDNA as it is present in the vast majority of solid tumors. It involves multiple chromosomal arms, providing an advantage of detecting chromosomal number changes, so that a score can be developed based on the cumulative number of changes in more than one locus in the genome (Douville et al. 2018). This strategy takes advantage of the presence of multiple different DNA fragments from different loci, effectively decreasing the amount of genome equivalents per locus required compared to when mutations are used. For this reason, aneuploidy could be detectable in a smaller volume of plasma. Although promising, current methods that rely on detecting changes in copy number have inferior analytical sensitivity than mutation detection methods (Lo et al. 2010; Jiang et al. 2015; Douville et al. 2018).

Size has also emerged as a difference between cfDNA and ctDNA. Studies have indicated that ctDNA is enriched in a fraction with an average size smaller than for cfDNA (Mouliere et al. 2011; Thierry et al. 2016). Evaluating mutations in this enriched fraction could help increase the signal-to-noise ratio for ctDNA. Choosing the appropriate biomarker to detect ctDNA requires knowledge of both characteristics of ctDNA, the amount anticipated in the sample, and the limits of the clinical application in terms of sensitivity and specificity.

3 Source of ctDNA

The ctDNA is derived from either the circulating tumor cells (CTCs) or the tumor cells located in the tumor bed. There are several pieces of evidence that support the tumor cells from the tumor bed as the principle source of ctDNA and not the CTCs. First, no cases have been observed where CTCs were detected in the absence of ctDNA. However, ctDNA is many times present in the absence of CTCs. In a direct comparison of DNA from the cellular fraction of the blood and ctDNA from the plasma of the same patients tested for the presence of somatic changes identified in the tumor tissue, in the cases in which ctDNA was detected, no CTCs were detectable with the identical assay, while the opposite was not true (Bettegowda et al. 2014). Second, in the cases where both CTCs and ctDNA are detected, the number of ctDNA genome equivalents is orders of magnitude higher than for CTCs. This discrepancy between the amount of ctDNA and the number of CTCs has been addressed both theoretically by comparing reported amounts of ctDNA and numbers of CTCs in patients with metastatic disease (Crowley et al. 2013; Thierry et al. 2016), and directly in the cellular fraction and plasma from the same blood draw. In the cases where both CTCs and ctDNA levels were detectable, the average number of mutant fragments in the plasma was >50-fold higher than analogous levels in CTCs (Bettegowda et al. 2014).

4 The Nature of ctDNA

In both physiological and pathological conditions, cfDNA is largely made up of short fragments of DNA. Initially, the size of the most abundant fragments of cfDNA in cancer patients was observed at ~180 bp (Giacona et al. 1998; Jahr et al. 2001). With the advent of next-generation sequencing approaches, the most prominent size of cfDNA in cancer patients was shown to be ~166 bp with a series of peaks every 10 bp at sizes smaller than 140 bp (Lo et al. 2010; Jiang and Lo 2016; Thierry et al. 2016; Wan et al. 2017). Although cfDNA is predominantly short, it is not devoid of large fragments sometimes measuring several kilobases in length. The size of cfDNA is thought to provide clues for its release mechanisms. Both apoptosis (Jiang and Lo 2016) and necrosis (Diehl et al. 2005; Thierry et al. 2016) have been proposed as the main mechanisms for the presence of ctDNA in the circulation. For example, the periodicity associated with the nucleosomes suggests that cfDNA is the product of apoptosis. Smaller fragments are consistent with the possibility that the DNA is degraded by nucleases or is the product of phagocytosis by macrophages of necrotic cancer cells. Large fragments might also be the product of necrosis.

In practice, the size of ctDNA is important for the development of methods for clinical assays. Adjusting the amplicon size of the *APC* gene from 1296 to 100 bp to assay both the wild-type (WT) and the mutant molecules representing the non-cancer-derived cfDNA and the ctDNA fraction, respectively, resulted in a five-

to 20-fold increase of total *APC* fragments (cfDNA) detected as the size decreased, while the fraction of mutant molecules (ctDNA) increased more than 100-fold over the same size range (Diehl et al. 2005). The increase in the fraction of *APC* mutant molecules detected in these studies demonstrated that tumor DNA was more degraded than normal DNA. Animal models for cancer where DNA can be easily distinguished based on human and mouse sequences, and studies in cancer patients support that much of the ctDNA is at <145 bp (Mouliere et al. 2013; Thierry et al. 2016). The integrity and size of ctDNA have required the development of methods that target short degraded fragments rather than larger fragments of DNA.

The second most important characteristic of the ctDNA for accurate detection is its half-life. Many reports have demonstrated that the half-life is short, on the order of minutes rather than hours, days, or weeks. Studies on pregnant women were the first to demonstrate that cfDNA is generally short-lived. The mean time for reduction of the fetal DNA by half was 16.3 min (range: 4–30 min) (Lo et al. 1999). In a study where the level of plasma Epstein-Barr virus (EBV) DNA in patients with nasopharyngeal cancer was monitored, the median half-life of plasma EBV DNA after surgical resection was 139 min (To et al. 2003). Based on one colorectal cancer patient whose plasma was sampled at multiple time points early after complete resection of the cancer, the half-life of ctDNA was 114 min (Diehl et al. 2008).

The half-life of ctDNA has especially important implications for cancer. A short half-life would make the detection of ctDNA an ideal diagnostic parameter as its disappearance and/or reappearance could be associated with efficacy of treatment and disease recurrence. Patients who undergo surgical resection exhibit a characteristic rise in ctDNA immediately following the procedure, but detection decreases dramatically shortly thereafter, while any future increases might indicate the presence of minimal residual disease and recurrence. This nature of ctDNA as a biomarker for tumor dynamics was shown in colorectal cancer patients undergoing treatment (Diehl et al. 2008). Subsequent studies have now shown that ctDNA could detect minimal residual disease and predict recurrence and response to therapy in a number of tumor types before imaging modalities making ctDNA a great biomarker for following the dynamic changes of the disease (Tie et al. 2016; Dawson et al. 2013; Chaudhuri et al. 2017; Pantel and Alix-Panabières 2017; von Bubnoff 2017).

5 Amount of DNA

The ability to detect and quantify mutant DNA molecules in the circulation is the basis of liquid biopsy. The calculated number of cfDNA molecules has varied from study to study, probably due to methodological and biological reasons. For the detection of ctDNA, the mutation allele fraction (MAF), or variant allele fraction

(VAF), is a critical parameter. Based on observed MAFs and assuming a half-life of 16 min, a large number of ctDNA molecules are still present. For example, a 30 g stage III colorectal cancer with *APC* mutations of 1.3% MAFs sheds 0.15% of tumor DNA in the circulation each day (Diehl et al. 2005). Analysis of patients with resistant to anti-EGFR inhibition metastatic colorectal cancers suggested that an estimated 44 million tumor cells with a *KRAS* mutation would result in at least one mutant *KRAS* fragment in one milliliter of plasma (Diaz et al. 2012).

While the MAF in the metastatic setting could be greater than 10% (Dawson et al. 2013; Guibert et al. 2018) enabling sensitive detection of ctDNA, the MAF in early detection and detection of minimal residual disease has been reported to be less than 0.1%. Sensitive tests should be able to detect an MAF of less than 0.01%. There are two problems that come up. One is technical. Many artifactual "mutations" are generated during the preparation of the ctDNA for analysis (enzymatic manipulation, amplification, and sequencing) and even during in silico analysis. As a result, the compound error rate of processing and analyzing ctDNA is higher than the analytic abilities of the available methods. This issue necessitates not only the development of methods with great analytical sensitivity, but also the incorporation of error correction steps, such as the inclusion of unique identifiers for each ctDNA fragment in the form of molecular barcodes (Kinde et al. 2011; Newman et al. 2014; Wan et al. 2017). The second problem is biological. Even with methods of this level of analytical sensitivity, in certain situations, like early detection of stage I tumors, the actual number of molecules present in the 5–10 mL of plasma that is usually used for mutation detection could be limited. For example, a 0.01% MAF for a mutation, which is one ctDNA fragment in 9999 cfDNA fragments encompassing the position of the mutation, translates to one mutant molecule in 30 ng of cfDNA. The amount of cfDNA available is limiting for the early detection of cancer. In a recent study focused on the detection of stage I and II pancreatic cancers, detection of *KRAS* or *TP53* mutations was limited by the number of mutant molecules present in the plasma and resulted in reduced sensitivity. Of the samples that were positive for *KRAS* mutations, 23% had less than 2 molecules per mL of plasma (Fig. 1) (Cohen et al. 2017). The availability of ctDNA molecules has been addressed in many situations, and it is the principle issue for many of the clinical applications of liquid biopsy for the detection of rare mutations. This has an unintentional consequence in the development of methods for the detection of ctDNA. While a method can be improved to have a greater analytical sensitivity by utilizing higher input or by including steps for enriching fractions of cfDNA enriched in ctDNA, the gains in analytical sensitivity may come at the expense of losing molecules affecting clinical sensitivity. The amount of ctDNA is obviously of paramount importance to clinical applications. Understanding the technical limitations and biological limitations should provide new avenues of how to circumvent them.

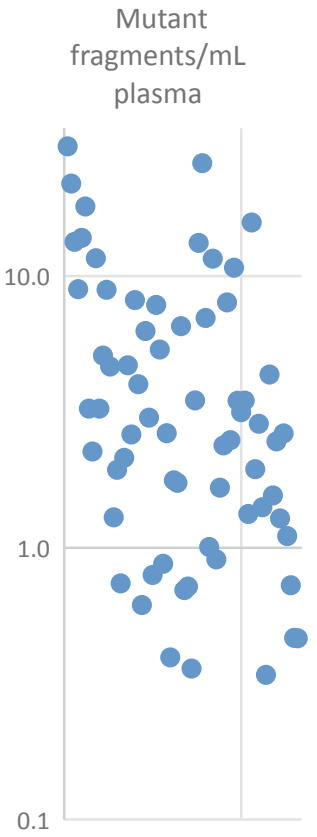

Fig. 1 Number of mutant fragments per mL plasma from patients with stage I and II pancreatic cancers. Each dot represents 66 patients positive for *KRAS* mutations. Fourteen and 23 patients had less than one and two DNA fragments per mL plasma, respectively. Data from Cohen et al. (2017)

6 Determination of the cfDNA/ctDNA Tissue of Origin

One of the foreseeable challenges in the field of detection of occult disease using ctDNA will be to identify the affected tissue/organ. For example, a screening test in asymptomatic populations would be much more informative if in addition to detecting a biomarker for the presence of cancer, it could also accurately predict the site of the tumor. Somatic mutations alone are not the answer, as many driver mutations are common among different cancers (Vogelstein et al. 2013). Furthermore, although it is possible that detection of multiple mutations in a single patient could provide information about the origin of cancer, the approach is not in general feasible given the rarity of ctDNA molecules. Therefore, other strategies need to be implemented to achieve tumor site prediction.

To begin to understand how to approach this aspect of assay design, investigators borrowed from the lessons learned in detecting fetal DNA in the plasma and sera of pregnant women (Lo et al. 1997, 1998a, b); they examined cfDNA in individuals with organ transplants where their genetic background was distinct from the transplanted tissue (Sun et al. 2015). Investigators subsequently explored the use of methylation or nucleosomal patterns in cfDNA to determine its tissue of origin (Lehmann-Werman et al. 2016; Snyder et al. 2016; Hao et al. 2017). Bisulfite sequencing was performed to identify methylated bases of plasma DNA from pregnant women, and cancer or transplant patients. The methylation profiles in ctDNA were compared to reference tissue-specific profiles (Sun et al. 2015). Similarly, methylation patterns in cfDNA correlated with tissue-specific cell death in several pathological conditions, including pancreatic cancer (Lehmann-Werman et al. 2016). Methylation patterns in ctDNA were also used to identify the site of metastasis in patients with colon cancer (Hao et al. 2017). Investigators have also used the boundaries of cfDNA fragments to map nucleosomal positioning and determine the tissue of origin based on known patterns (Snyder et al. 2016). They reasoned that the fragment length distributions with a dominant peak at 167 bp support a model in which cfDNA fragments are protected from nuclease cleavage in association with nucleosomal proteins. Using this approach, they could effectively generate a footprint of protein–cfDNA interactions which would theoretically correlate with the tissues of origin. In a cohort of a few cancer patients, the patterns of nucleosome spacing revealed significant non-hematopoietic contributions to the cfDNA that many times matched the tissue origin of the cancer. A combination of somatic and epigenetic changes might thus make it possible to detect a cancer and determine its site of origin.

A different approach is to use other types of markers in addition to ctDNA. Recently, the use of an artificial intelligence algorithm integrating values from ctDNA and protein biomarkers not only increased the sensitivity in detecting cancer, but also helped in illuminating possible locations of the primary tumor (Cohen et al. 2018).

7 Release and Clearance

The presence of cfDNA has been attributed to many sources, either exogenous from infections or transplantation, or endogenous due to cell death from apoptosis, necrosis, NETosis, or particles released from cells, such as exosomes. More recent publications contain more comprehensive and detailed information (Aucamp et al. 2018; Thierry et al. 2016). Multiple causes lead to these mechanisms responsible for cfDNA release. For example, cfDNA is increased with sepsis, inflammation, aging, exercise, and cancer among other physiological and pathological conditions. There are also consequences from the presence of cfDNA. cfDNA released from activated neutrophils triggers blood coagulation, for example, after chemotherapy, and it has been reported as a prognostic factor for disseminated intravascular coagulation, a process common among cancer patients (Swystun et al. 2011; Kim et al. 2015).

What is the mechanism for release of ctDNA, and is it different than the mechanism for release of cfDNA? As we discussed earlier, size has led investigators to propose apoptosis/necrosis as one of the mechanisms for ctDNA release. Necrotic or apoptotic DNA was thought to simply leak into the circulation from dead cells, but different scenarios involving immune cell types might explain how ctDNA ends up in the circulation. Studies performed in cell culture have shown that necrotic cells do not simply leak DNA into the circulation; rather, macrophages are needed in vivo for DNA from necrotic cells to be released into the blood (Choi et al. 2005). Macrophages arrive at the necrotic areas to clean up the dead material which includes the DNA. The DNA is digested into the smaller fragment size in macrophages, which eventually release their contents into the bloodstream. A second mechanism involving neutrophils might explain the presence of cfDNA in the circulation in diverse pathological conditions. Neutrophil extracellular traps (NETs), which are complexes of protein and intriguingly released DNA, have been studied in a few pathological situations. In cancer, NETs have been identified within the tumor bed, and their formation has even been shown to be triggered by metastatic breast cancer cells (Park et al. 2016). However, it is not clear if and how much this mechanism contributes to the amount of ctDNA.

Exosomes, and other pieces of cells surrounded by parts of a cell membrane (I affectionately call them trashosomes), have been shown to contain ctDNA. Exosomes themselves have been explored as biomarkers for detecting cancer. Most protocols for the isolation of ctDNA will include, inevitably, DNA from the exosomes, and many studies reporting on ctDNA quantities include ctDNA from the exosomes.

The mechanism of clearance for ctDNA has not been well studied, but most likely it is cleared the same way as cfDNA. It appears that a combination of clearance through the kidneys and uptake from liver and spleen accounts for the elimination of ctDNA. Tumor-derived DNA that has been cleared in urine, from distal organs to the kidney, is shorter in size than ctDNA suggesting further degradation. Clearance will also be affected by the physiological or pathological state of the patient. Investigators have injected labeled single-stranded DNA into mice and then examined organs for levels of radioactivity. Studies examining the clearance of ssDNA at increasing concentrations of DNA in animals demonstrated that liver ($\sim$90%) is the major site for removal (Emlen and Mannik 1978) with kidneys taking up 2–5% and spleen even less. Detection in lung and skin was negligible. Liver could be saturated with increasing concentrations of DNA, however; and in this case, spleen uptake increased.

There are still many unanswered questions about the causes and mechanisms of cfDNA release limiting our understanding of its biology. Studies of ctDNA in clinical samples have provided some clues about the relationship of ctDNA presence to the state of the cancer that shed it to the circulation.

8 Biological Considerations of ctDNA

The inability to detect ctDNA in the plasma of every cancer patient might not strictly be due to suboptimal sensitivities of the available analytical methods. Other factors related to the biology of ctDNA and our understanding of its release into the circulation could also subvert its detection. There is a gross correlation between amount of ctDNA and tumor burden. On the other hand, there are observations that indicate gaps in our knowledge of ctDNA biology and our ability to predict accurately its rate of release in the circulation. This critical knowledge, if obtained, could help us develop better clinical applications and interpret their results.

Previous studies have shown that the total DNA concentration in the plasma of cancer patients is often elevated (Leon et al. 1977; Sozzi et al. 2001). This increase has been supported by subsequent studies in individuals with advanced cancers (Diehl et al. 2005; Newman et al. 2014). But, the ctDNA fraction alone cannot account for the increase in total cfDNA, indicating that in addition to tumor cells, other non-tumor cells, presumably from the tumor bed, or cells infiltrating the tumor die at the same time. This increase in cfDNA could be better rationalized when an individual is under therapy, assuming that the therapeutic approach causes death of both cancer and non-cancer cells. The general increase of cfDNA, however, will dilute the signal from ctDNA. What is important for the detection of ctDNA is the number of fragments present in the plasma and the percentage of mutant fragments.

Studies have shown that ctDNA in patients with advanced tumors from different tumor types is not detected with equal sensitivity and is most likely due to the different numbers of ctDNA molecules shed (Bettegowda et al. 2014). In a separate study, the amount of cfDNA varied between non-metastatic tumor types. Patients with hepatocellular carcinoma had on average more cfDNA than other tumor types, resulting in more ctDNA fragments per mL of plasma. However, the actual fraction of ctDNA was not higher than in other tumor types (Fig. 2) (Cohen et al. 2018). Differences in the sensitivity of detection correlating with tumor type have also

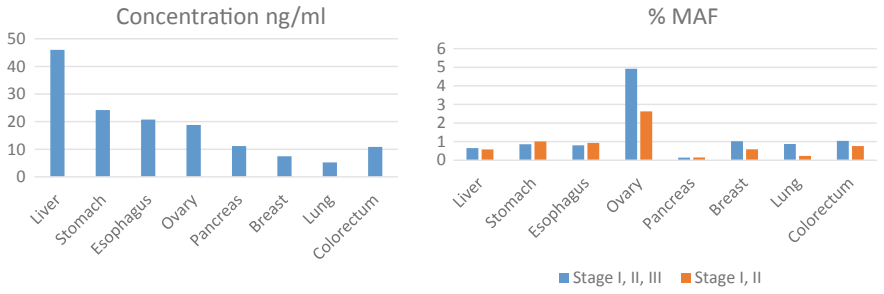

Fig. 2 Panel A shows average concentration of cfDNA from different tumor types prepared with the same protocol within the same study. Panel B shows the % MAF from the same samples indicating that increased amount of cfDNA does necessarily correspond to more % MAF

been noted in other studies. Lung squamous cell carcinomas were detected with greater sensitivity than lung adenocarcinomas of the same stage (Abbosh et al. 2017). Different tumor types have a different biology, different levels of aggressiveness, and different rates of turnover which are all characteristics most likely reflected in the levels of ctDNA released.

Even within the same tumor type, there are differences in the amount of released ctDNA. Head and neck squamous cell carcinomas were detected with different sensitivities when they were segregated by location. Tumors located in the oropharynx, larynx, and hypopharynx were detected with higher sensitivity than those located in the oral cavity (Wang et al. 2015a). These results suggest that the location of the tumor could affect the amount of ctDNA released into the blood. Is this because of physical characteristics, like proximity of the tumor to the circulation, or due to not well-understood differences between the tumors? Lack of sensitive detection of CNS tumors has also been blamed on the location of the cancers beyond an intact blood-brain barrier, in some cases leading to the use of CSF as the bodily fluid for the detection of tumor released DNA (Wang et al. 2015b).

As we discussed earlier, the volume of the tumor will influence the amount of ctDNA. There is a correlation between stage and amount of ctDNA fragments, as later-stage cancers tend to be larger than early-stage cancers. Whether ctDNA levels in general are exactly proportional to systemic tumor burden cannot easily be determined, largely because there is not a good, alternative way to measure tumor burden. Measurements from imagining cannot accurately identify the proportions of cancer cells, inflammatory cells, and non-neoplastic cells within the tumor (Li et al. 2007). The range of mutant fragments per mL of plasma varies significantly from tumor to tumor no matter what the stage is (Bettegowda et al. 2014; Phallen et al. 2017; Cohen et al. 2018). Even within tumors of the same type and stage, such as stage I and II pancreatic cancers, the correlation between size and detectable ctDNA was not good (Cohen et al. 2017). It has recently been well documented that ctDNA can detect minimal residual disease ahead of imaging with a very reasonable analytical sensitivity close to 0.002% MAF (Tie et al. 2016; Chaudhuri et al. 2017). These metastatic lesions are not larger than early-stage localized primary cancers. One reason for this distinction is that detection of minimal residual disease is not performed blinded. The more typical approach is a personalized test that requires the detection of a known genetic alteration, usually a mutation in a driver gene present in the primary tumor. In the early detection setting, there is no prior knowledge of the genetic alteration which makes the task more difficult. However, in proof-of-principle studies where tissue was available and personalized assays were feasible, the correlation between size and sensitivity in the detection of ctDNA was still not proportional. Adenomas of the colon with lesions equivalent or larger than those of colorectal carcinomas rarely had detectable ctDNA (Diehl et al. 2005).

These observations provide evidence that the presence of ctDNA correlates better with the invasive cancers. It was thought that necrotic cells are the main source of the ctDNA, and the more the necrosis in the cancer, the more ctDNA will be detectable (Diehl et al. 2005). Indeed, large metastatic tumors have large areas of

necrosis, which is not the case with early-stage localized cancers. In the lung cancer TRACERx study, there was a correlation between the extent of necrosis and the amount of ctDNA (Abbosh et al. 2017). In the same study, ctDNA levels were also correlated with an increased Ki67 proliferation index and increased FDG avidity on PET imaging. A better correlation of cfDNA with parameters other than tumor burden, such as metabolism, has also been observed in other studies (Morbelli et al. 2017). Thus, the abundance of ctDNA may have more to do with the state of the tumor than its actual physical size. These results perhaps also provide an important clue indicating that metastatic lesions are different from the primary lesions; they may have higher turnover, more necrosis, or a not yet well-understood mechanism that results in the ctDNA release at levels that are easier to detect than when the cancer is localized. ctDNA may ultimately not be a sensitive marker for detecting benign or indolent lesions with ramifications for their underdiagnosis.

9 Biology and Interpretation of Genetic Alterations in ctDNA

Liquid biopsy is ideal for the detection of multiple metastatic lesions present within then same individual (Misale et al. 2012; Diaz et al. 2012) and enables monitoring of resistance (von Bubnoff 2017). But, how do we know that the mutations are really derived from tumor cells? This point is extremely relevant in cases where the tumor itself is not available. Concordance between the driver mutations present in the tumor tissue and the mutations present in the plasma from the same individuals provided initial validation that the ctDNA indeed represented the mutations within the tumor and established that the liquid biopsy approach works. However, the concordance has not been always perfect. In two recent studies with multiple tumor types, the concordance between mutations in primary tumors and ctDNA mutations varied from 90% (Cohen et al. 2018) to 82% (Phallen et al. 2017). One explanation for this discrepancy is the presence of heterogeneity; however, this tumor characteristic does not account for the majority of the cases, as heterogeneity in localized cancers is very rare when it is driver mutations that are being evaluated. Another explanation is that many of the mutations are technical artifacts, and the analysis algorithms cannot discriminate them from real mutations. Technical advances in mutation detection and algorithms for their identification have decreased the rate of artifactual mutations (Kinde et al. 2011; Phallen et al. 2017; Cohen et al. 2018; Chaudhuri et al. 2017; Newman et al. 2016). Finally, it is also possible that the mutations derive from occult cancers.

Analytical specificity and sensitivity do not always translate to a method conducive to clinical applications. True-positive mutations in driver genes could also be due to clonal proliferation unrelated to cancer (Genovese et al. 2014; Xie et al. 2014). The most common type of these mutations is present in clonal hematopoiesis of indeterminate potential (CHIP). This has been a large issue, especially in early detection of cancer. To eliminate these mutations as cancer-derived, both the

cellular fraction and the plasma from the same blood are tested for their presence. A mutation that is present in both fractions is presumed to originate from non-cancer cells. However, the specificity of this assay has not yet been established. In addition, there are other clonal proliferations that have mutations exactly like the somatic driver mutations in cancer cells. Recently, it has been shown that endometriosis lesions have mutations identical to those found in ovarian cancers. *KRAS* mutations have been even identified in arteriovenous malformations (Anglesio et al. 2017; Nikolaev et al. 2018). It is not clear if mutations from these conditions are represented in the circulation and at what levels. All these situations could contribute to false positives making the interpretation of the presence of mutations more complicated. Longitudinal prospective studies for monitoring cancer could shed light on some of these situations and help in the interpretation of a result. The prevalence of CHIP-related mutations increases with age. The same is true not only for clonal expansion, but for non-clonal mutations (Hoang et al. 2016). Such mutations present a challenge because many of the current studies include individuals that are usually at an age when screening is assumed to be recommended, >50 years. Perhaps, studies in younger individuals can at least provide information about the characteristics of a test in a biological background where the potential for CHIP is reduced.

Epilogue

ctDNA is no longer buried in the annals of molecular research. The field has come long way, and clinical applications utilizing ctDNA have even gained FDA approval. ctDNA detection and its applications are moving fast. However, mechanisms of release and/or increase of ctDNA in the circulation remain somewhat ill-defined; it is still not clear if some tumors do not shed ctDNA, or that the analytical methods are not sensitive enough. Can such knowledge help us to safely and transiently increase the levels of ctDNA, so we can detect it more efficiently?

There are several proposals to increase sensitivity, including collecting more blood or interrogating larger areas of the genome. Each has advantages, but also disadvantages. Some of them are practical, and some based on biology. More blood certainly will increase sensitivity, but may not be acceptable in some situations and result in reduced compliance. Large genome areas could provide the advantage of having multiple trials to identify a signal and potentially avoid stochastic events that can be missed when a single mutation is used as the biomarker. On the other hand, this approach will increase background errors and create issues with the interpretation of the results. The false discovery rate increases with increased numbers of bases included in the analysis as well (Wan et al. 2017). In addition, such an approach might rely on the analysis of non-driver mutations making the interpretation of the results difficult, especially in older individuals who already might harbor cfDNA detectable mutations that are not of cancer origin. Such approaches assume that the issue lies in the inefficiency of the analytical approach to capture

and interrogate fragments with genetic alterations derived from cancer cells. However, this may not be always the case, or the desired sensitivity may not be achievable within acceptable levels of specificity.

To this end, other approaches are being explored. We mentioned earlier the possibility of combining somatic mutations and epigenetic changes as biomarkers for the detection of cancer. In a study, mentioned earlier, the sensitivity in detecting head and neck cancers varied based on the anatomical location of the cancer. To test the synergy between different bodily fluids in increasing the sensitivity of detecting mutations, both saliva and blood were collected from people with head and neck cancer. The sensitivity of detecting cancers present in the oral cavity and hypopharynx was 100 and 67%, respectively, when DNA isolated from saliva was used, while the sensitivities were 80 and 100% when ctDNA prepared from blood was used (Wang et al. 2015a). Similar results have been observed for ovarian cancer in samples collected from Pap smears and plasma from the same individuals (Wang et al. 2018). While all these combinations include tumor-derived DNA, a recent study explored the combination of ctDNA and protein markers for the early detection of cancer, providing very encouraging results (Cohen et al. 2018). We can imagine that other types of analytes, like metabolites, could augment sensitivity when combined with ctDNA.

There are many studies trying to address the issues associated with ctDNA detection. But, there are not any systematic studies yet to address if location within an organ, vascularization, or the number of mitotic figures correlates better with the amount of released ctDNA. The combination of improved methods for the detection of cancer and improved understanding between the relationship of ctDNA release and the pathological state of the cancer could help in providing better quality information for the management of cancer patients, by providing more significant quantitative and qualitative measurements of ctDNA.

An intersection of current knowledge, technical capabilities, and the requirements of clinical applications (some requiring superb sensitivity and some requiring superb specificity), even with our current status of incomplete knowledge, could help develop better ways of managing cancer patients. Detecting ctDNA for clinical applications should not be viewed in the absence of other parameters or as the sole determinant of a clinical decision. Studies, especially prospective, with accurate clinical information about the participants and their tumors, performed under IRB-approved protocols, will reveal relationships between ctDNA, its potential synergy with other analytes or other modalities, the status of the patients, and tumor characteristics. Deeper investigation into the answered questions about ctDNA will help us relate fundamental biological questions to real clinical situations.

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Enrichment and Analysis of ctDNA

Pauline Gilson

1 Introduction

In routine clinical practice, besides tissue biopsy being considered for long time as the gold standard source for cancer molecular analysis although providing only a snapshot of the tumor molecular signatures (Diaz and Bardelli 2014), liquid biopsy has progressively emerged as a minimally-invasive and "real-time" surrogate for accessing the tumor genome (Ilié and Hofman 2016). Liquid biopsy represents all acellular body fluids that give access to tumor-derived materials. Tumor DNA can be profiled in different body fluids including urine (Reckamp et al. 2016), sputum (Mao et al. 1994), saliva (Wang et al. 2015), stool (De Maio et al. 2014), pleural (Kimura et al. 2006), cerebrospinal fluids (De Mattos-Arruda et al. 2015) and blood that is currently the more studied. Circulating tumor DNA (ctDNA) are found in bloodstream of cancer patients as a part of cell-free DNA (cfDNA), resulting from tumor cell apoptosis, necrosis or active release (Thierry et al. 2016; Stroun et al. 2001). Molecular alterations identified in ctDNA are reflective of cell heterogeneity within the tumor and in all distant tumor sites (Siravegna et al. 2017) and have already shown clinical utility for diagnosis and prognosis, treatment decision-making, therapy-response monitoring, tracking clonal evolution and emergence of resistance (Siravegna et al. 2017). Detection and quantification of ctDNA are technically challenging and requires sensitive and specific techniques considering that ctDNA are highly fragmented by nature and "diluted" by cell-free

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DNA from non-malignant origin thus representing as few as 0.01% of total cfDNA in early-stage cancers (Diaz and Bardelli 2014; Diehl et al. 2005; Haber and Velculescu 2014; Diehl et al. 2008a).

Herein, we discuss pre-analytical requirements for optimal ctDNA analysis. We also provide an overview of current technologies to exploit liquid biopsies with their potential clinical applications. Finally, we briefly summarize recent technical advances for high throughput and ultrasensitive detection of ctDNA.

2 Optimal Pre-analytical Practices

Pre-analytical phase includes blood collection, sample processing and DNA isolation. Each of these steps may affect the final ctDNA yield and stability and subsequent measurements highlighting the need for rigorous and standardized procedures to translate cfDNA analysis into clinical practice (Bronkhorst et al. 2015).

2.1 Matrix of Choice for ctDNA Analysis

Studies reported that cfDNA concentrations are 2-24 times higher in serum than in plasma samples (Jung et al. 2003; Vallée et al. 2013). Higher levels of cfDNA in serum are mainly consistent with non-malignant DNA release from white blood cell lysis during the clotting process thus reducing the relative amount of ctDNA in a high non-tumoral background and potentially leading to a false negative result (Lee et al. 2001; Chan et al. 2005). Serum also shows more inter-patient variations in cfDNA levels compared to plasma (Bronkhorst et al. 2015). Based on these observations, plasma represents a more suitable matrix for ctDNA analysis.

2.2 Collection Tube

When using plasma, EDTA anticoagulant is privileged over other anticoagulants given that it confers a better stability of cfDNA concentrations following venepuncture (Warton et al. 2014). However, the use of EDTA tubes does not preclude the need for plasma isolation within the day of collection as genomic DNA release from leukocytes significantly increases beyond this time (Lam et al. 2004). Heparin, that likely exhibits PCR-inhibiting properties (Beutler et al. 1990) and does not restrain the activity of cfDNA-degrading endonuclease (Lu and Liang 2016), is often contraindicated for ctDNA analysis.

For practical reasons and easier sample collection, transportation and preparation in clinical research context, specialized cfDNA-formulated blood collection tubes containing fixative agents for leukocyte stabilization and cfDNA integrity preservation may be used. The PAXgene™ tubes (Qiagen, Germany) and cell-free

DNA™ blood collection tubes (Streck Inc., Omaha, USA) show equivalent cfDNA preservation for up to 7 days at room temperature or 30 °C daytime while the cfDNA collection tubes (Roche Diagnostics, Germany, previously found in Ariosa) are less efficient at elevated temperature (Nikolaev et al. 2018).

2.3 Sample Processing

A recent external quality assessment program emphasizes a high inter-laboratory variability in pre-analytical procedures including the requisite sample volume, the plasma preparation and the DNA isolation procedure (Haselmann et al. 2018). A major prerequisite for optimal cfDNA analysis consists of a complete removal of cellular components from plasma fraction to avoid the risk for genomic DNA contamination (El Messaoudi et al. 2013). In this context, plasma needs to be ideally isolated within the first 6 h following blood drawing (El Messaoudi et al. 2013). Low-speed blood centrifugation (800 g) is not sufficient to ensure efficient cell-free plasma preparation (Swinkels et al. 2003). A two-step centrifugation protocol is optimal for initial sample processing whereby a first whole-blood centrifugation at 1600 g for 10 min is performed to get rid of most blood cells followed by a second plasma-sample centrifugation at 16,000 g for 10 min to improve plasma purity (El Messaoudi et al. 2013; Chiu et al. 2001).

2.4 Plasma Storage Conditions

Before DNA extraction, plasma samples may be stored for 2 weeks at −80 °C without modifying plasma cfDNA content (Chan et al. 2005). More than 3 freeze and thaw cycles affect the integrity of cfDNA in plasma samples while DNA extracts are more resistant to such conditions (Chan et al. 2005). Therefore, it is suited to aliquot plasma samples in order to minimize freezing and thawing and proceed to DNA isolation as soon as possible.

2.5 CfDNA Isolation

For downstream ctDNA analyses, different current approaches for DNA isolation can be used: spin column-based, magnetic bead-based, and phase isolation methods (Lu and Liang 2016). Depending on the method used, differences in terms of DNA yield, purity and efficiency of small DNA fragment recovery are observed and should be taken into account for cfDNA analysis. Phase isolation techniques (such as phenol-chloroform procedure) achieve significantly higher yields of DNA and recover a broader range of DNA fragment sizes (including ctDNA small fragments) however they are more complex and time-consuming than other methods (Fong et al. 2009). Numerous studies compare the available commercial ready-to-use kits for DNA extraction and purification. The QIAamp DNA Blood™ Mini Kit

(Qiagen, Hilden, Germany) is able to recover 82–92% of cfDNA from serum, however, this kit is designed for large fragments (>200 pb) enrichment making it not suitable for ctDNA analysis (Lu and Liang 2016). The QIAamp Circulating Nucleic Acid™ Kit (Qiagen) and the Plasma/Serum Circulating DNA™ kit (Norgen Biotek, Thorold, Canada) are reported among the most efficient column-based methods to provide high cfDNA amounts and favor the capture of small DNA fragments (Perakis et al. 2017). According to Kloten and coll., magnetic bead-based system need to be preferred over a spin column one for the enrichment of low-sized DNA fragments (Kloten et al. 2017; Fleischhacker et al. 2011). Automation [using either the Maxwell Rapid Sample Concentrator™ from Promega (Madison, USA); QIAsymphony™ and QIAcube™ from Qiagen or MagNa Pure™ from Roche (Meylan, France)] can be considered as a robust and reproducible option for this pre-analytical step. The appropriate input plasma volume for cfDNA extraction depends on the isolation method and the kit used but a consensus is established for volume ranging from 2 to 3 mL (Devonshire et al. 2014).

A recent time-saving approach that does not require the plasma DNA extraction prior qPCR analysis significantly shows higher cfDNA amounts compared to conventionally obtained DNA eluates and avoid any fragment size bias (Breitbach et al. 2014).

2.6 CfDNA Storage Conditions

For mutations detection, cfDNA extracts should be stored 9 months at most at −20 °C or −80 °C and a maximum of three freezing-thawing cycles is suitable (El Messaoudi et al. 2013). For ctDNA quantitation and fragmentation, long-term storage is limited to 3 months at -20 °C.

3 Technical Approaches for ctDNA Analysis

Technologies for ctDNA analysis can be divided into two categories: PCR-based and new generation sequencing-based methods. Both regroup targeted approaches that aim to detect specific anomalies in a predefined set of genes and untargeted approaches that screen the genome without a priori in order to identify new genomic alterations.

3.1 PCR-Based Assays (Table 1)

PCR per se does not discriminate between wild-type and mutant sequences and amplifies both with the same efficiency, hampering the detection of low-abundance DNA mutant variants. Moreover, the use of a DNA polymerase which is intrinsically prone to make replication errors every 1000 bases limits the sensitivity of this

Table 1 PCR-based methods

Targeted/non-targeted approach	Technique	DNA input or volume of plasma/blood required	DNA isolation required (yes/no)	Median turnaround time (including DNA isolation if needed)	Analytical sensitivity (% mutant to wild-type abundance ratio)	Quantitative results	Multiple samples per run (yes/no)	Multiplexing (number of targets assessable in meantime)	Type of alterations detected	References
Targeted approaches	SuperARMS EGFR mutation detection™ kit	10 mL of blood 15 ng/reaction	Yes	4 h	0.2–0.8%	No	Yes	Yes (41 targets)	SNVs, indels	(Little 2001; Spindler et al. 2012; Cui et al. 2018)
	Cobas EGFR mutation™ test v2	150 ng of DNA (50 ng of DNA/reaction) or 2 mL of plasma	Yes	<4 h	0.1–0.8%	No	Yes	Yes (42 targets)	SNVs, indels	(Malapelle et al. 2017; Keppens et al. 2018)
	Idylla™	1 mL of plasma	No	<2 h	$\leq$1–5%	No	No	Yes ($\leq$30 molecular targets)	SNVs, indels	(Janku et al. 2015; Janku et al. 2016; Jacobs et al. 2017)
	Intplex™	2 mL of plasma	Yes	48 h	0.004–0.014% (1/10,000)	Yes	Yes	Yes ($\leq$10 molecular targets)	SNVs, indels	(Mouliere et al. 2014; Thierry et al. 2014)
	PAP	100–200 ng DNA	Yes	/	1/10,000–1/100,000	Yes	Yes	Yes	SNVs, indels	(Liu and Sommer 2000; Liu et al. 2006)

(continued)

Table 1 (continued)

Targeted/non-targeted approach	Technique	DNA input or volume of plasma/blood required	DNA isolation required (yes/no)	Median turnaround time (including DNA isolation if needed)	Analytical sensitivity (% mutant to wild-type abundance ratio)	Quantitative results	Multiple samples per run (yes/no)	Multiplexing (number of targets assessable in meantime)	Type of alterations detected	References
Non-targeted approach	Enhanced-ice-COLD-PCR	25 pg–25 ng input	Yes	4 h	0.01%–<0.05%	Semi-quantitative	Yes	Yes, variable	SNVs, indels	(Tost 2016; How-Kit et al. 2014; Mauger et al. 2016)
Targeted approaches	qMSP	1–4 mL plasma 100 ng of DNA for submission to bisulfite treatment	Yes	Total duration: ~24 h (Bisulfite conversion of DNA in 1 h–18 h, depending on the amount and quality of DNA input)	1%	Semi-quantitative	Yes	Yes	DNA methylation profile	(Herman et al. 1996; Wielscher et al. 2015; Ellinger et al. 2008; Hauser et al. 2013; Ng et al. 2011; Sasaki et al. 2003)
	QX100 ddPCR™ system	10 mL of blood samples	Yes	5 h	0.005–0.01%	Yes	Yes	Yes	SNVs, indels, CNV	(Hindson et al. 2011; Hindson et al. 2013; Sanmamed et al. 2015; Tsao et al. 2015; Gray et al. 2015)

(continued)

Table 1 (continued)

Targeted/non-targeted approach	Technique	DNA input or volume of plasma/blood required	DNA isolation required (yes/no)	Median turnaround time (including DNA isolation if needed)	Analytical sensitivity (% mutant to wild-type abundance ratio)	Quantitative results	Multiple samples per run (yes/no)	Multiplexing (number of targets assessable in meantime)	Type of alterations detected	References
Targeted approaches	BEAMing	2 mL of plasma	Yes	48 h–72 h	0.01% (1/10,000)	Yes	Yes	Yes ($\geq$ 10 mutations)	SNVs, indels	(Diehl et al. 2006; Li et al. 2006; Denis et al. 2017; Dressman et al. 2003)
	Raindrop Plus™ system	10 mL of blood samples	Yes	Total duration: ~7 h (4 h 15 excluding DNA isolation)	1/200,000–1/1,000,000	Yes	Yes (8 samples/run)	Yes ($\leq$ 10 targets, use of 2 colors and varying concentrations of probes)	SNVs, indels	(Pekin et al. 2011; Taly et al. 2013; Yu et al. 2017)
	Naica™ system for crystal digital PCR	3 mL of plasma	Yes	Total duration: ~5 h (2 h 30 excluding DNA isolation)	0.09%	Yes	Yes (up to 12 samples per run)	Yes (3–5 colors)	SNVs, indels, CNV	(Jovelet et al. 2017; Madic et al. 2016)
	Biomark™ system with the BioMark 12.765 Digital Array Chip	2–6 mL of blood	Yes	/	0.1%	Yes	Yes (12 samples per run)	Yes	SNVs, indels	(Basu 2017; Yung et al. 2009)
	Quantstudio 3D Digital PCR System™	8–10 ml of blood	Yes	~7 h	0.04–0.1%	Yes	1 sample per chip	Yes (2 targets per chip, 2 colors)	SNVs, indels, CNV	(Feng et al. 2018; Masago et al. 2018)

Abbreviations

BEAMing Beads, Emulsion, Amplification and Magnetics; *CNV* copy number variations; *ddPCR* droplet digital PCR; *indels* insertions/deletions; *PAP* Pyrophosphorolysis-activated polymerization; *qMSP* Methylation-specific qPCR; *SNP* single nucleotide polymorphisms; *SNV* single nucleotide variations

approach. In the last years, new PCR-based strategies (such as partitioning or mutant-selective enrichment) have been developed to circumvent these issues and make ctDNA analysis currently reachable and competitive with NGS approaches.

3.1.1 The Amplification Refractory Mutation System—qPCR (ARMS-qPCR)

ARMS-qPCR associates a real-time quantitative PCR methodology with selective amplification of minority (mutant) alleles. ARMS-qPCR also known as allele-specific PCR (ASPCR) or PCR amplification of specific alleles (PASA) may be applied for the identification of any known mutations involving single point mutations or microdeletions (Lo 1998). The use dof allele-specific primers enables the amplification of the target allele if present in a multi-allele mixture without the amplification of non-targeted alleles (Little 2001). PCR primers can discriminate a single nucleotide change given that any nucleotide mismatch toward the 3′ end of the primer hinders the PCR amplification. ARMS-qPCR has the advantage to potentially differentiate heterozygotes and homozygotes for either allele using distinct labeled-primers for the mutant and the wild-type alleles (Newton et al. 1989). Many commercial kits employing ARMS-qPCR offer a multiplex and time-saving approach convenient for molecular testing in clinical practice.

- **The SuperARMS EGFR Mutation Detection™ kit (Amoy Diagnostics Co., Xiamen, China)**

The SuperARMS EGFR Mutation Detection™ kit is implemented by Cui et al. for the multiplexed detection of up to 41 *EGFR* mutations in plasma cfDNA from lung adenocarcinoma patients with an analytical sensitivity that ranges from 0.2 to 0.8% (Little 2001; Spindler et al. 2012).

- **The Cobas EGFR mutation™ test v2 commercial kit (Roche Diagnostics, Meylan, France)**

The Cobas EGFR mutation™ test v2 commercial kit is intended for the simultaneous qualitative detection of 42 common *EGFR* mutations in matched tumor tissue and plasma samples (Malapelle et al. 2017). For such purpose, oligonucleotide probes selective for each target mutation with both a reporter fluorescent dye and a quencher dye attached are used. Three reactions in separate wells are required to obtain the complete mutational status in the tested regions of the *EGFR* gene. This assay represents the first FDA-approved blood-based companion test for osimertinib in lung cancer patients with disease progression. Kepens et al. show a limit of detection as low as 100 copies of T790M-mutant DNA/mL of plasma using 25 μL of DNA input per reaction (Keppens et al. 2018).

• **Idylla™ platform (Biocartis, Mechelen, Belgium)**

Idylla™ is a fully-automated allele-specific real-time PCR technology that integrates all steps in a microfluidic cartridge (Janku et al. 2015). Idylla™ enables the concomitant qualitative detection of up to 30 different mutations in less than 2 h. Given that the disposable cartridge is preloaded with all reagents needed for the assay and is closed once the sample is inserted, it avoids the risk of cross-contamination (Luca et al. 2017). Several cartridges are available depending on the clinical context. For example, Idylla ctBRAF mutation™ test is designed for the specific detection of *BRAF* V600 mutations in plasma-derived cfDNA from multiple advanced cancer patients with a sensitivity and a specificity estimated at 73% (95% CI, 0.60–0.83) and 98% (95% CI, 0.93–1.00) respectively (Janku et al. 2016). Comparative study shows a high concordance for *BRAF* mutation status in plasma cfDNA between Idylla™ system and standard dPCR assays (BEAMing, ddPCR) (Janku et al. 2016).

• **Intplex™ (DiaDx, Les Matelles, France)**

Intplex™ is an allele-specific blocker qPCR-based approach specifically developed for the detection of already known point mutations in cfDNA (Mouliere et al. 2014; Thierry 2016). Briefly, 2 sets of primers are used to target sequences of less than 100 bp and distant from nearly 300 bp (Fig. 1) (Mouliere et al. 2014). The first primer pair is designed to display low melting temperature and amplify the specific mutated region while the second pair targets a wild-type sequence. A blocking oligonucleotide harboring a phosphate group in 3′ is added into the PCR mix to avoid the non-specific elongation of the wild-type sequences thus improving the

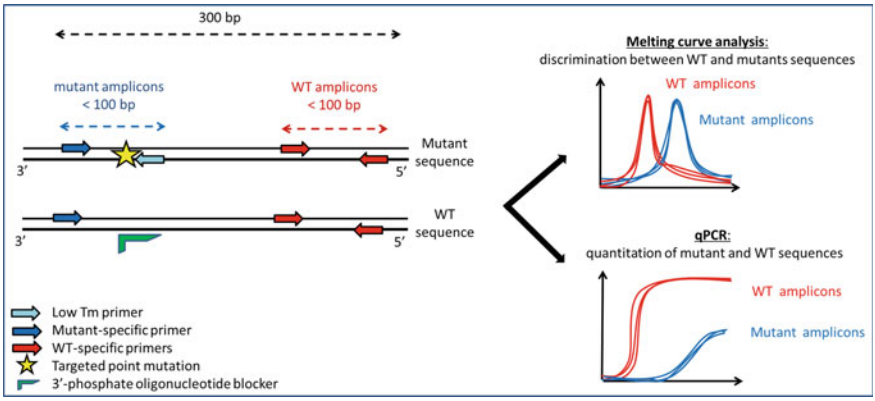

Fig. 1 Principle of the Intplex™ system. Two primer pairs targeting mutant (in blue) and wild-type (in red) regions respectively are designed to generate amplicons with similar size. A low melting temperature primer system is used to amplify the mutant sequence while a blocking oligonucleotide (in green) targeting the WT sequence avoids the non-specific elongation of the wild-type sequences

specificity of the assay (Mouliere et al. 2013). Mutational status is determined by assessing the concordance with a positive control through melting-curve analysis. The tumor mutation load is determined according to the Cq from qPCR analysis. Overall, Intplex™ provides 5 distinct parameters: the cfDNA concentration (including non-malignant cfDNA and ctDNA), the detection of a known point mutation, the ctDNA concentration, the proportion of ctDNA (concentration of ctDNA sequences/concentration of WT sequences) and the cfDNA integrity index (concentration of 300 bp-sequences/concentration of 100 bp-sequences) (Thierry 2016). This technique was implemented for the detection of *KRAS* and *BRAF* hotspots mutations in plasma-derived cfDNA from colorectal cancer patients and shows sensitivity that ranges from 0.004 to 0.014% (Thierry et al. 2014).

- **Pyrophosphorolysis-activated polymerization (PAP)**

Pyrophosphorolysis-activated polymerization is an allele-specific PCR-derived approach that relies on the use of an oligonucleotide (*P**) blocked with a 3′ dideoxynucleotide (Fig. 2) (Liu and Sommer 2000). Once specifically annealed to its complementary target DNA sequence, *P** is activated by pyrophosphorolysis through the removing of the terminal dideoxynucleotide enabling extension to occur by DNA polymerization. This PCR-derived technology is highly specific

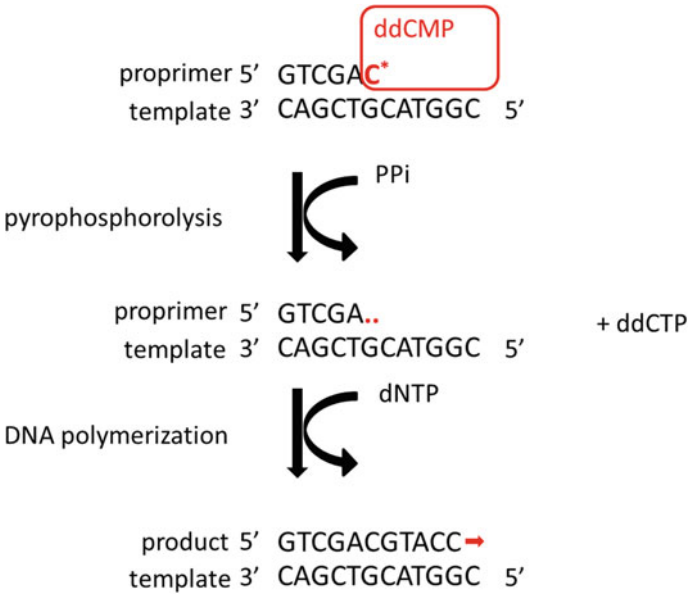

Fig. 2 Schematic representation of the Pyrophosphorolysis-activated polymerization. A proprimer blocked with a 3′ dideoxynucleotide specifically binds to its complementary target sequence. The polymerase removes the dideoxynucleotide by pyrophosphorolysis thus allowing the primer to extend by DNA polymerization

since it differentiates 2 sequences that differ by only a single nucleotide (Madic et al. 2012). This technique has been recently optimized for multiplexing detection (Liu et al. 2006).

3.1.2 Cold-PCR

Co-amplification at lower denaturation temperature (COLD)-PCR is an enrichment method that allows the preferential amplification of minority sequences in a complex mixture of wild-type and mutated species independently of the nature of the mutation and the location along the amplicons (Li et al. 2008). In cases of low-abundance mutated variants, COLD-PCR can replace classical PCR and be combined with most downstream detection technologies (such as Sanger sequencing, pyrosequencing, NGS-based approach, real-time PCR or HRM analysis) in order to significantly enhance their sensitivity by up to 100-fold (Li et al. 2008; Milbury et al. 2011a; Li et al. 2009). The strength of this technique is that it magnifies all minority mutant alleles regardless of whether mutations are known or not (Milbury et al. 2009). COLD-PCR exploits slight differences in amplicon melting temperature (T_m) between wild-type and mutation-containing sequences. The T_m for amplicon sequences up to 200 bp changes of 0.2–1.5 °C depending on the presence of single nucleotides changes or small deletions and their location along the sequence (Lipsky et al. 2001). A critical denaturation temperature (T_c) is specifically defined for each DNA sequence as the temperature just lower than the T_m below which the amplicons are unlikely denatured and the PCR efficiency strongly decreases in consequence. By fixing the PCR denaturation temperature at T_c, amplicons with one or more nucleotides are preferentially denatured and amplified over wild-type sequences throughout the course of PCR.

Many formats of COLD-PCR (full-, fast-, ice- (improved and complete enrichment-), enhanced-ice, and temperature-tolerant COLD-PCR) with variable performance and enrichment specificity have been developed. For example, Full-COLD-PCR enables the identification of all mutation types including T_m-decreasing, T_m-neutral, and T_m-increasing mutations. However, it provides a modest enrichment potential (by 3–10 fold) and is an excessively long five-step PCR approach (Mauger et al. 2017). Fast COLD-PCR is a simplified protocol that achieves higher mutation enrichments (by 10–100 fold) but only identifies mutations with T_m lower than that of the wild-type sequences (such as G:C > A:T or G:C > T:A) (Mauger et al. 2017). The more recent Ice-COLD-PCR combines the advantages of both full- and fast-approaches (Milbury et al. 2011b).

3.1.3 Methylation-Specific QPCR (QMSP)

In recent years, DNA methylation profile has emerged as a promising epigenetic biomarker for cancer diagnosis, prognosis and therapy-response monitoring thus requiring new strategies to distinguish the methylated from the unmethylated DNA CpG islands (Lissa and Robles 2016). One of the most common approaches consists of bisulfite treatment combined with qPCR-based methods. Bisulfite reagent converts unmethylated cytosines into uracils and randomly generates single-stranded breaks while leaving most methylated cytosines intact to be detected by subsequent

PCR amplification (Herman et al. 1996). The conversion rate is however variable according to the available commercial kits and may lead to misleading results. Besides, the chemical conversion per se leads to DNA fragmentation and degradation thus decreasing DNA yield and the approach has a low multiplexing capability. An alternative non-bisulfite method consists of enzymatic DNA digestion using methylation-sensitive restriction endonucleases (MSRE) that solely cleave unmethylated-cytosine residues followed by qPCR assays that amplifies residual uncutted methylated DNA (Wielscher et al. 2015; Ellinger et al. 2008; Hauser et al. 2013; Ng et al. 2011).

3.1.4 Digital Polymerase Chain Reaction (dPCR)

Digital PCR represents a third-generation PCR technology introduced in the 1990s by Vogelstein and Kinzler (Sykes et al. 1992; Vogelstein and Kinzler 1999). It converts the exponential analog response from conventional PCR techniques into linear digital signal, thus allowing endpoint absolute quantification of nucleic acids. Complex DNA mixtures (such as those isolated from biological samples) are partitioned into multiple individual compartments and separate real-time PCR reactions are performed using fluorescent probes that specifically target mutant or wild-type species (Fig. 3) (Perkins et al. 2017; Perez-Toralla et al. 2015). Based on

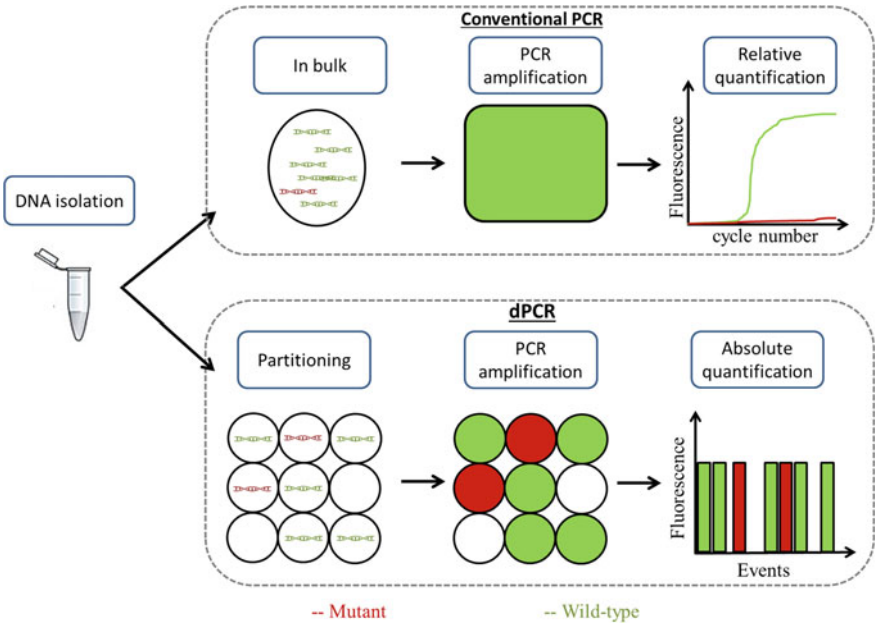

Fig. 3 Comparison between conventional PCR and dPCR systems. In conventional PCR, a bulk PCR reaction is performed thus masking mutant targets (in red) in abundant wild-type sequences (in green). In dPCR approach, the sample is partitioned into individual compartments for multiple PCR reactions in parallel and digital counting gives rise to an absolute quantification. This strategy increases the relative abundance of rare mutant alleles allowing their detection in a wild-type background

Poisson statistics, either one or zero DNA molecule is initially present within a partition in such a way that all amplicons generated in each fraction derive from a single DNA template. Through this strategy, it increases the relative abundance of rare occurrences and allows their quantification by calculating the ratio of positive to negative answers with no need to rely on standard calibration curves (Yi et al. 2017). The sensitivity of dPCR techniques is variable (in a range from 0.005 to 0.04%) mainly depending on the number of partitions that can be analyzed (Perkins et al. 2017; Yi et al. 2017). This limiting dilution of DNA samples is crucial to identify rare mutants diluted in a large wild-type background and confers increased tolerance to PCR inhibitors that can be found in biological fluids (Baker 2012). dPCR systems also hold promise for the detection of less than 30% difference in gene expression (Baker 2012), the measurement of tumor-associated copy number variations (Hindson et al. 2011; Whale et al. 2012) and miRNAs (Hindson et al. 2013). There are two different approaches for the compartmentalization: the use of multiple physical partitions for sample splitting or the generation of water-in-oil emulsions to sequester DNA fragments into individual droplets.

ddPCR (Droplet Digital PCR)

- **The QX100/QX200™ ddPCR system (Bio-Rad, Marnes-la-Coquette, France)**

The QX100/QX200™ ddPCR devices partition each DNA sample in up to 20,000 nanoliter droplets monodispersed within immiscible oil so that each one contains either 0 or 1 sequence of target and background DNA (Hindson et al. 2011, 2013). Thousands of droplets generated act as isolated dPCR microreactors with Evagreen chemistry or TaqMan hydrolysis probes to discriminate the mutated sequences from the wild-type background. Up to 96 DNA samples can be processed in meantime. This system can be employed to detect and quantify *BRAF* V600E mutations even at low fractional abundance (approaching 0.005–0.01%) in the plasma from melanoma patients with a clinical specificity of 100% (Sanmamed et al. 2015; Tsao et al. 2015). The tracking of *NRAS* resistance mutations is also possible by ddPCR with a specificity higher than 73% (Gray et al. 2015).

- **BEAMing (Beads, Emulsion, Amplification, and Magnetics)**

The BEAMing technology employs a combination of emulsion digital PCR with magnetic beads and flow cytometry (Fig. 4). Pre-amplified DNA templates are partitioned in a water-in-oil emulsion generating millions of individual droplets each of which containing a single copy of DNA and one magnetic microbead with specific primers covalently coated to the surface (Diehl et al. 2006; Diehl and Smergeliene 2013). During emulsion dPCR process in each droplet, the PCR products are generated and remain attached to the beads, then the emulsion is broken and the beads are purified through magnetic procedure. Two distinct fluorescent probes hybridize to the captured DNA fragments and enable the

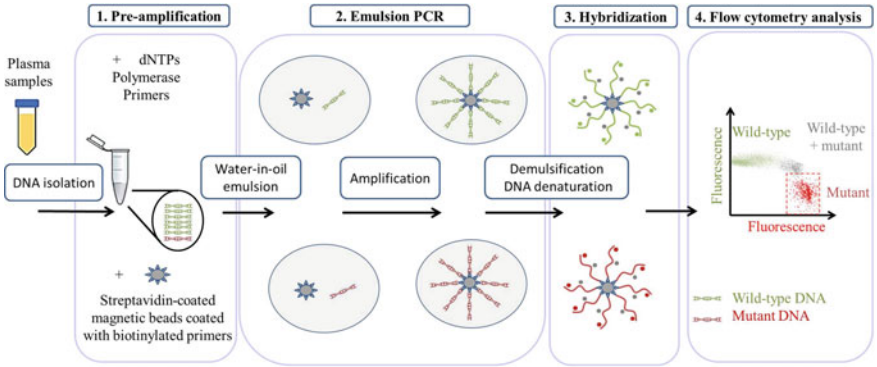

Fig. 4 Principle of the BEAMing™ system. Mutant (in red) and wild-type (in green) fragments contained in a DNA sample are both pre-amplified by multiplex PCR (Diaz and Bardelli 2014). A water-in-oil emulsion creates millions of droplets, each containing single DNA sequence and individual streptavidin-coated magnetic bead coated with specific biotinylated primers. Multiple PCR reactions are performed in each droplet generating millions of identical DNA templates attached to each bead (Ilié and Hofman 2016). The emulsion is broken and the beads are recovered using a magnet. Captured DNA fragments are denatured and hybridized with fluorescent probes specific for mutant and wild-type sequences (Reckamp et al. 2016). Beads carrying wild-type and mutant DNA are finally distinguished by flow cytometry (Mao et al. 1994)

distinction of beads carrying wild-type and mutant DNA by flow cytometry. The BEAMing digital PCR has shown efficacy in detecting known ctDNA mutations even at a fractional abundance of 0.01% in the wild-type gene (Diehl et al. 2008b). For example, the OncoBEAM™ RAS CRC Kit (Sysmex inostics, Hamburg, Germany) is able to analyze a panel of 34 hotspot mutations in exons 2, 3 and 4 of the KRAS and NRAS oncogenes (García-Foncillas et al. 2017) and shows a 93% agreement in mutation calling with standard tissue-based procedures (Vidal et al. 2017; Schmiegel et al. 2017).

- **The RainDrop Plus™ system (RainDance Technologies, Lexington, United States)**

In RainDrop Plus™ system, DNA samples are loaded into a one-time use 8-panel microfluidic chip and partition in up to 10 millions picoliter-sized droplets each encapsulating only a single target DNA regardless of the DNA concentration in the input. The RainDrop Plus™ technology thus generates millions of separated single-molecule PCR enabling the detection of a mutated allele diluted in 200,000 wild-type DNA copies (Pekin et al. 2011).

Such hyper-compartmentalized picodroplet platform gives access to multiplexing in which each partition contains multiple molecular-detection assays. The use of 2 fluorescent Taqman probes and varying concentrations of them allows the detection of up to 10 mutations in a single assay (Zhong et al. 2011). To demonstrate,

Taly et al. developed a multiplex dPCR approach in millions of picoliter droplets for the simultaneous detection of 7 hotspot mutations in codons 12 and 13 of the *KRAS* gene in 2 assay panels from the plasma of colorectal cancer patients (Taly et al. 2013). This system gives a 74% concordance with standard tissue-based testing methods and allows the detection of low-frequency mutant variants less than 1%. In the same way, Yu et al. devised 2 multiplex dPCR panels to screen for 19DEL and T790M mutations (4-plex panel) and L858R and T790M mutations (5-plex panel) in plasma samples from advanced non-small cell lung cancer patients using 2 individual microfluidic chips (Yu et al. 2017). This approach confers an overall concordance of 80% with standard-of-care tissue testing and its sensitivity and specificity were 90.9 and 88.9% for 19DEL, 87.5 and 100% for L858R, 100 and 93.8% for T790M mutations respectively. This approach has the advantage to detect even low variants down to approximately 0.01–10% mutation abundance (Yu et al. 2017).

- **The Naica™ system for Crystal Digital PCR (Stilla Technologies, Villejuif, France)**

A microfluidic chip generates the partitioning of DNA complex sample into a 2D array of 30,000 individual droplets (named droplet crystal). PCR amplification at endpoint is directly performed on-chip then the droplets that contain amplified DNA targets are distinguished from those that did not using three different fluorescent detection channels.

This system has the advantage to integrate all steps of the process in a single consumable and the mean turnaround time is nearly 2 h 30 with no more than 5 min hands-on-time. Owing to its 3-color detection, Crystal Digital PCR technology offers the possibility to perform multiplexing assays. Jovelet et al. developed a such approach for the concomitant detection and quantification of wild-type *EGFR* and 4 different *EGFR* sensitizing and resistance mutations (del19, L858R, L861Q, and T790M) in plasma ctDNA from advanced *NSCLC* patients with variant allele fraction as low as 0.09% (Jovelet et al. 2017).

Microfluidics dPCR or Chamber Digital PCR (cdPCR)

Microfluidics dPCR combines lab-on-a-chip and digital PCR approaches. Through a system of channels and valves, DNA samples can be partitioned into several independent reaction chambers on a disposable microfluidic device.

- **The microfluidic-chamber-based Biomark dPCR™ (Fluidigm Corporation, Les Ulis, France)**

The Biomark 12.765 Digital Array Chip™ (Fluidigm Corporation, Les Ulis, France) is composed of 12 panels each containing 765 chambers with a 6 nL volume per unit thus simultaneously performing 9180 PCR reactions in a single PCR run (Basu 2017). An expanded device displaying 36,960 independent chambers of 0.85 nL volume allocated in 48 panels of 770 chambers is also commercially available. This high-throughput microfluidics digital PCR platform

requires less sample and reagents than other systems. This system has been used for the detection of L858R and in-frame del19 EGFR mutations in plasma from lung cancer patients with a sensitivity as low as 0.01% (Yung et al. 2009).

- **The microwell chip-based QuantStudio 3D dPCR™ (Thermofisher Scientific, Illkirch, France)**

The Quantstudio 3D Digital PCR™ System contains a high-density nanofluidic chip with up to 20,000 independent reaction wells (0.8 nL per well) and is reported to be sensitive and capable of detecting T790M *EGFR* mutations present in ctDNA from non-small cell lung cancer patients at low level (<0.5%) (Feng et al. 2018). Heyries et al. develop an extended approach with a microfluidic megapixel digital PCR that allows the sample partitioning into a million picoliter PCR chambers by means of an immiscible fluid for the detection of only a single nucleotide variant in 100,000 wild-type sequences (Heyries et al. 2011).

3.2 Next-Generation Sequencing-Based Methods (or Massive Parallel Sequencing-Based Methods) (Table 2)

Although PCR-based approaches have been successfully adapted for ctDNA analysis, they are limited by low multiplexing ability and are mostly not designed to interrogate de novo mutations considering the need for prior knowledge of molecular targets. By this way, they can miss the detection of non-hotspot but otherwise clinically relevant anomalies. The historical and widely available Sanger sequencing is compromised in their utility for ctDNA genotyping considering its lack of sensibility (approaching 10–20%), the time-consuming, its cost and its low throughput (Cheng et al. 2016; Loeb et al. 2003). These restrictions may be overcome by NGS technologies allowing the highly sensitive molecular testing of multiple genomic regions in a single assay (Malapelle et al. 2016). NGS methodology supports a wide range of applications including DNA mutations profiling, determination of tumor mutation burden (Davis et al. 2017), identification of chromosomal aberrations and rearrangements (Aguado et al. 2016; Leary et al. 2012), gene expression screening (Wang et al. 2018) and detection of epigenetic changes (Warton et al. 2014). It can either offer ctDNA targeted analysis or expanded screening with variable sequencing efficiencies and costs depending on the method used. In all cases, it can be divided into four steps: DNA library generation, DNA fragment amplification, sequencing, and raw data bioinformatics analysis. The two major currently commercialized NGS platforms are those produced by Illumina (San Diego, CA, USA) and Life Technologies (ThermoFisher Scientific, Waltham, MA, USA) (Lee et al. 2013).

Table 2 NGS-based methods

Targeted/nontargeted approach	Technique	DNA input or volume of plasma/blood required	DNA isolation required (yes/no)	Analytical sensitivity (% mutant to wild-type abundance ratio)	Quantitative results	Targets	Type of alterations detected	References
Targeted sequencing	AmpliSeq	2 mL plasma 1–100 ng DNA	Yes	>2% (2% for SNPs and 5% for indels)	Yes	Panel of genes	SNVs, indels	(Rothé et al. 2014; Kaisaki et al. 2018; Butler et al. 2016)
	Safe-SeqS	3 ng DNA	Yes	0.1%	Yes	Panel of genes	SNVs, indels	(Kinde et al. 2011, 2013)
	TAm-Seq	$\leq$ 2 ml plasma	Yes	>2%	Yes	Panel of genes	SNVs, indels	(Forshew et al. 2012; Dawson et al. 2013)
	Capp-Seq	7–32 ng DNA	Yes	0.02%	Yes	Panel of genes	SNVs, indels, CNV, rearrangements	(Newman et al. 2014; Bratman et al. 2015)
	TEC-Seq	5-250 ng of cfDNA	Yes	0.05-0.1%	Yes	Panel of genes	SNVs, indels	(Phallen et al. 2017)
	Guardant360™	5–30 ng DNA two 10 mL-tubes of blood draw	Yes	<0.1%	Yes	Panel of 73 genes	SNVs, indels, CNV, rearrangements,	(Lanman et al. 2015)
	FoundationOne™ liquid	two 8.5 mL-tubes of blood draw	Yes	>0.5% for SNVs, indels and rearrangements, $\geq$20% for copy number variations	Yes	Panel of 70 genes	SNVs, indels, CNV, rearrangements, MSI status	

(continued)

Table 2 (continued)

Targeted/nontargeted approach	Technique	DNA input or volume of plasma/blood required	DNA isolation required (yes/no)	Analytical sensitivity (% mutant to wild-type abundance ratio)	Quantitative results	Targets	Type of alterations detected	References
Non-targeted sequencing	WES	50 ng-1ug DNA	Yes	>1–3%	Yes	all annotated exons, microRNA, long intergenic noncoding RNA, UTRs	SNVs, indels, CNV, rearrangements	(Murtaza et al. 2013; Warr et al. 2015; Majewski et al. 2011; Klevebring et al. 2018; Takai et al. 2015; Butler et al. 2015; Manier et al. 2018; Meienberg et al. 2015)
	WGS	250 ng DNA	Yes	1%	Yes	whole genome	SNVs, indels, CNV, rearrangements, chromosomal aberrations	(Leary et al. 2012; Chan et al. 2013; Heitzer et al. 2013)

Abbreviations

CAPP-Seq Cancer Personalized Profiling by deep Sequencing; *CNV* copy number variations; *indels* insertions/deletions; *MSI* microsatellite instability; *Safe-SeqS* Safe-Sequencing System; *SNP* single nucleotide polymorphisms; *SNV* single nucleotide variations; *TAm-Seq* Tagged-amplicon deep sequencing; *TEC-Seq* Targeted error correction sequencing; *UTRs* untranslated regions; *WES* whole-exome sequencing; *WGS* whole-genome sequencing

3.2.1 Targeted Sequencing

Targeted sequencing does not cover the whole spectrum of cancer-specific alterations but employs focused gene panels covering the clinically relevant targets so that each is redundantly sequenced thousands of times (ultra-deep sequencing). Compared to whole genome (WGS) or whole exome sequencing (WES), targeted NGS confers the advantages to improve the coverage depth and sensitivity on selected regions of the genome associated with a reduced cost and a facilitation of data analysis.

Targeted sequencing relies on two major approaches that differ from the targeted enrichment method used before sequencing: amplicon- and capture-based methods. Multiple technological and bioinformatical (in silico error suppression) adaptations have been proposed over time in order to maximize sequencing performance and/or limit the cost.

Amplicon-Based Methods

Amplicon-based methods consist of the PCR amplification of specific genomic regions using specific primer pairs.

- **AmpliSeq**

The AmpliSeq is an amplicon-based enrichment method that can generate up to thousands of target amplicons for the analysis of SNP, indels, gene copy variations or gene fusions starting from either DNA or RNA samples. The ampliSeq panels offer a pool of oligonucleotide primers to perform a multiplex PCR-based library preparation. Ready-to-use or custom panels are commercially available to interrogate multiple regions across a gene (such as the Ion AmpliSeq™ BRCA1/2 Panel for Ion Torrent (Life Technologies) or the AmpliSeq™ BRCA1/2 Panel for Illumina), or focus on specific hotspot mutations (as the Ion AmpliSeq™ Cancer Hotspot Panel v2 (Life Technologies) or the AmpliSeq™ Cancer HotSpot Panel v2 (Illumina)). All these panels are originally designed for tumor biopsy testing however they have been successfully applied for biopsy-free ctDNA analysis (Hirotsu et al. 2017; Rothé et al. 2014; Kaisaki et al. 2018). Major technological challenges for the use of AmpliSeq are the high detection level (>2%) and the background noise.

Pecuchet et al. improved the detection performance of the technology by coupling AmpliSeq with a low-cost statistical approach (Pécuchet et al. 2016). The base-position error rate (BPER) method assesses the variability and background noise of each base position to decrease the risk of false-positive results. For a given sample, the BPER approach quantifies the error rate base to base and compares it with the minimal mutated allele frequencies to establish if alterations are true or not. The BPER correction could be used to identify single nucleotide variations and indels spanning more than 2 bp with mutant allele fractions as low as 0.003 and 0.001% respectively (Pécuchet et al. 2016).

- **Safe-Sequencing System (Safe-SeqS)**

The Safe-SeqS method assigns a unique molecular identifier (UMI, also termed barcodes or index) to each DNA molecule (Kinde et al. 2011). The amplification of the labeled-DNA sequences creates UMI families with daughter molecules having identical sequence to the original template (if no PCR amplification or sequencing artifact occurs). After redundant sequencing of the amplicons, variants are considered real if at least 95% of the reads group with the same unique identifier carry the same mutation. Such strategy extends the number of alterations simultaneously detected and decreases the error rate due to replication or sequencing bias promising a higher sensitivity in mutation detection (a mutation could be detected among a background of 5000 to 10^9 wild-type nucleotides) (Kinde et al. 2011). This technology has already been applied for the detection of tumor-related alterations in ctDNA from GIST or colorectal cancer or in papanicolaou smears from ovarian and endometrial cancer patients (Tie et al. 2015; Fredebohm et al. 2016; Kinde et al. 2013).

- **Tagged-amplicon deep sequencing (TAm-Seq)**

TAm-Seq approach combines a two-step amplification and sequencing of large genomic regions from low counts or fragmented DNA as observed in plasma samples (Forshew et al. 2012). Primers are designed to cover large sequences in short overlapping amplicons. A pre-amplification step using multiple target-specific primers enables the conservation of all templates present in the sample. Multiple single-plex PCR is then performed to selectively amplify specific sequences. Molecular barcodes are finally added to the end of the generated amplicons before pooling and sequencing. Forshew et al. who first described the technique to detect *TP53* mutations in plasma from high-grade serous ovarian cancer patients, show a sensitivity and a specificity higher than 97% (Forshew et al. 2012). A technical limitation of Tam-Seq that needs to be improved on is the allelic frequency threshold to detect mutant alleles of >2% much higher than for most of dPCR-based methods.

In 2018, Gale et al. described an enhanced TAm-Seq™ (eTAm-Seq) approach that associates efficient library preparation and statistically-based analysis algorithms to significantly decrease the detection sensitivity down to 0.02% and enable the detection of specific genomic regions in up to 35 genes (Gale et al. 2018). Beyond the identification of single nucleotide variants and short indels, eTAm-Seq can also report copy number variations.

Capture-Based Methods

Capture-based methods enrich the samples for genomic regions of interest by hybridization to complementary biotinylated oligonucleotides followed by capture with streptavidine-coated beads.

- **Cancer Personalized Profiling by deep Sequencing (CAPP-Seq):**

CAPP-Seq is an ultrasensitive technology that associates hybrid affinity capture of multiple regions of interest with deep sequencing. It relies on the use of selector probes to uncover mutations in a large majority of patients (>95%) with a specific cancer type with no need for individual optimization (Newman et al. 2014). The CAPP-Seq selector is designed using a multi-step bioinformatics approach and publicly available tumor sequencing data to target only genomic regions that are recurrently mutated in the cancer of interest. It is necessary to strike a good balance between the number of targets and the size of the selector in order to maximize the sequencing coverage in each position and limit the cost. CAPP-Seq can profile single nucleotide variants, indels, rearrangements, and copy number alterations and has been first applied to non-small cell lung cancers reaching a 96% specificity and an analytical sensitivity down to 0.02% (Newman et al. 2014). It can be generalized for any cancer types with known recurrent alterations in early- or late-stage disease.

Newman et al. significantly improve the detection sensitivity of the CAPP-Seq technology using an in silico strategy for the suppression of sequencing bias while adopting molecular barcoding for the recovery of rare mutant templates at copy ratios greater than 4:100,000 (Newman et al. 2016). This integrated digital error suppression (iDES)-based approach is first successfully implemented in non-small cell lung cancers with a sensitivity and specificity of 90% and 96% respectively (Newman et al. 2016).

- **Targeted error correction sequencing (TEC-Seq)**

TEC-Seq is a highly sensitive and specific approach that substantially decreases the sequencing error rate down to one false positive per 3 million bases sequenced (Phallen et al. 2017). TEC-Seq captures the most frequent alterations in cancer driver genes coupled with a deep sequencing of the targeted sequences for a 30,000-fold coverage of the regions of interest. The use of dual index adapters attached to DNA sequences during the library formation (exogenous barcodes) and the consideration of start and end positions of DNA fragments (endogenous barcodes) enables the identification of identical molecules among the duplicated sequences. Tumor-specific alterations are defined when an identical redundant sequence change is found in multiple distinct molecules. Due to its low sensitivity and minute levels of background error noise, TEC-Seq provides opportunities to detect mutations even in early-stage patients and minimal residual disease estimation (Phallen et al. 2017).

- **Guardant 360™ digital sequencing test (Guardant Health, Redwood City, CA, USA)**

Guardant 360™ is a commercial cfDNA hybrid-capture NGS testing based on Digital Sequencing™ that analyzes an expanded 73-gene panel for point mutations, gene fusions, indels and gene amplifications using two 10 mL-tubes of whole blood

(Lanman et al. 2015). In the digital sequencing™ workflow, each strand of double-stranded DNA fragments are tagged with oligonucleotide heptamer barcodes. A post-sequencing bioinformatics process analyzes both strands of the individual molecules thus enabling accurate base calls and eliminating most false positives. Through this strategy, Guardant Health argues a 100-fold decrease in error rate compared to conventional NGS approaches thus conferring to Guardant 360™ an ultra-high specificity (>99.9999%) with no false-positive mutation call in nearly 1.6 million bases sequenced and an analytic sensitivity as low as 0.1% (Lanman et al. 2015).

- **FoundationOne™ liquid (Foundation Medicine Inc., Cambridge, MA)**

FoundationOne™ liquid is a liquid biopsy NGS assay that can be applied to multiple advanced solid tumor patients for the screening of clinically relevant genomic alterations in 70 genes. As the previously cited Guardant 360™, this capture-based sequencing method is coupled with computational algorithms to improve the accuracy of base calls. FoundationOne™ liquid is claimed to have a high degree of sensitivity (>99% and 95% respectively) for point mutations, indels, gene rearrangements at >0.5% allelic fraction and copy number alterations with $\geq$ 20% tumor fraction. This broad molecular profiling assay also offers the possibility to report high Microsatellite Instability statuses.

3.2.2 Non-targeted Sequencing

Advances in the development of sequencing technologies have driven novel perspectives for genomic analyses. Broad DNA sequencing is emerging to expedite the discovery of new molecular driver mutations or cancer-predisposing alterations outside frequently mutated sites. It holds great promise for the identification of novel therapeutic targets or biomarkers clinically useful for cancer screening, diagnosis, prognostic and treatment. In the last decade, there have been a growing number of proof-of-concept studies for the analysis at the exomic (whole-exome sequencing, WES) (Murtaza et al. 2013; Jones et al. 2008; Parsons et al. 2008; Sjöblom et al. 2006; Jones et al. 2009) or genomic scale (whole-genome sequencing, WGS). However, many limitations restrain the use of WGS and WES for clinical routine. These technologies require large amounts of input DNA and are generally less sensitive than targeted NGS or digital PCR-based methods. These technologies also show substantial variability in sequencing efficiency across the genome leading to a lack of coverage in many regions of interest and potential missed variants. Besides, they generate overwhelming amounts of data that require storage facilities and sophisticated bioinformatics filtering techniques and software to be analyzed. Finally, WGS and WES approaches are still expensive and raise ethical issues due to the accidental discovery of germline mutations.

WES

Whole-exome sequencing focuses on the coding sequences that represent only 1% of the whole genome (about 30 Mb) but harbor around 85% of the disease-causing

mutations (Rabbani et al. 2014). Targeted content may be expanded to contain functional non-protein coding sequences (microRNA, long intergenic noncoding RNA, untranslated regions UTRs) (Warr et al. 2015). It provides a cost-effective alternative to WGS with lower costs for both sequencing and storage and analysis of the data. As the probes for exome capture process are designed based on the information provided by gene annotation databases, only known well-annotated coding genes are captured and analyzed (Majewski et al. 2011). The major exome capture platforms include the SeqCap™ EZ Human Exome library (Roche Nimblegen Inc, Madison, WI, USA) (Klevebring et al. 2018), the SureSelect Human All Exon kit (Agilent Technologies) (Takai et al. 2015; Butler et al. 2015), the TruSeq Exome Enrichment (Murtaza et al. 2013) or the Nextera Rapid Capture Exome kit (Manier et al. 2018) (Illumina) (Warr et al. 2015; Meienberg et al. 2015). As an example of application, Murtaza et al. successfully exploit WES for the identification of treatment resistance mutations in cfDNA from advanced cancer patients (Murtaza et al. 2013).

WGS

WGS interrogates the whole genome that includes the coding and noncoding regions (around 3.4 Gb). Given that the capture process is skipped in the WGS workflow, this technique has the advantage over WES to more efficiently cover coding exons (especially GC-rich regions) and sequence all the exome including some regions that can be missed by the exome capture process. WGS offers promising possibilities to determine DNA copy number variations and chromosomal aberrations (Leary et al. 2012; Chan et al. 2013; Heitzer et al. 2013).WGS is much more expensive than WES (Schwarze et al. 2018) and generates a volume of data 100 times bigger than the already large amounts obtained by WES (Majewski et al. 2011) making it currently less exploited for clinical care.

4 Conclusion

Liquid biopsy represents a promising alternative to the invasive and hardly repeatable tissue biopsy for the management of cancer patients. Analysis of ctDNA is however challenging considering the minute amounts present in the body fluids and requires optimized and standardized pre-analytical procedures for further optimal ctDNA detection and determination. Many approaches with variable performance and design are proposed for ctDNA analysis and should be considered depending on the clinical context. PCR-based approaches offer an ultra-high sensitivity, however, they are focused on specific known mutations and is mostly adapted for monitoring purposes. Contrariwise, NGS-based assay allows a broad screening of mutations and are suitable for de novo mutation discoveries, theradiagnostics or identification of resistance mutations. Until now, PCR assays and targeted sequencing assays have been favored because of their low costs, short

turnaround time and ethical reasons. However, in the near future, WGS and WES could be more used as their cost is still decreasing over time with the rapid evolution of the sequencing systems and bioinformatics.

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Capturing Tumor Heterogeneity and Clonal Evolution by Circulating Tumor DNA Profiling

Florian Scherer

1 Introduction

The understanding of genetic and epigenetic processes that drive cancer is becoming increasingly important for accurate diagnosis and choice of therapies. Major advances in sequencing technologies facilitate comprehensive analysis of cancer genetic landscapes and have led to the identification of hundreds of driver mutations across different tumor types (Hiley et al. 2014). Yet, researchers and clinicians are still at the beginning of understanding the full extent of tumor heterogeneity and the meaning of subclonal evolution for cancerogenesis and disease progression (Perdigones and Murtaza 2017). These two biological phenomena have serious implications for the clinical management of cancer patients. First, they might explain why groups of patients respond to standard cancer treatment, while the identical therapeutic strategy fails in other patients diagnosed with the exact same cancer type (Tannock et al. 2016). Second, capturing tumor heterogeneity has become increasingly important to identify patients most likely to benefit from targeted therapies and those who develop resistance mechanisms after a certain time (Do et al. 2015; Horak et al. 2017; Kurtz et al. 2019). Furthermore, robust characterization of mutational profiles might allow classification of patients into distinct risk groups at diagnosis and detection of genetic processes that lead to changes in tumor behavior over time, such as histological transformation. Finally, the evolution of genetic landscapes within a cancer significantly hampers the clinicians' ability to select effective treatment at disease recurrence, because tumor genotypes at the time of progression usually do not fully resemble diagnostic tumor genotypes.

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Repeated tumor biopsies to monitor clonal evolution over time and to fully evaluate tumor heterogeneity at major disease milestones are desirable, but they are often highly invasive procedures, bear the risk of major intra- and postsurgical complications, are inconclusive in some cases, and usually do not allow comprehensive assessment of the patient's genetic landscape (Manoj et al. 2014; Jain et al. 2006; Malone et al. 2015). The use of circulating tumor DNA (ctDNA) as a clinical biomarker has the potential to revolutionize detection and characterization of genetic profiles in cancer patients. Cancer cells release fragments of DNA into the bloodstream, where they can be noninvasively and repeatedly sampled by simple blood draws throughout the course of a disease (="liquid biopsy") and easily isolated from the blood plasma or serum compartment (Wan et al. 2017; Kurtz et al. 2018). Circulating tumor DNA, as part of the cell-free DNA (cfDNA) pool, reflects both the current tumor burden and the genetic landscape of all tumor sites in the patient's body, and thus represents an ideal alternative for comprehensive profiling of tumor genotypes. These characteristics of ctDNA potentially enable the full characterization of different levels of tumor heterogeneity and identification of tumor subclone evolution during treatment and towards disease progression (Fig. 1) (Newman et al. 2014; Scherer et al. 2016a; Chabon et al. 2016a; Bettegowda et al. 2014; Yeh et al. 2017b; Dawson et al. 2013; Kwapisz 2017; Kurtz et al. 2019; Chaudhuri et al. 2017).

In this chapter, we first discuss the technical aspects and requirements for robust and accurate noninvasive ctDNA genotyping (section "*Technical aspects of noninvasive tumor genotyping*"). Then, we explore the role of ctDNA profiling for detection and characterization of distinct tumor heterogeneity types, which can be categorized as follows (Fig. 1): (i) ***intratumor heterogeneity***: describes the genetic landscape within a single tumor manifestation, which is often not fully captured by partial tumor tissue sampling such as core needle biopsies (section "*Capturing intratumor and intrapatient heterogeneity by ctDNA profiling*"); (ii) ***intrapatient or spatial tumor heterogeneity***: represents the genetic landscape between distinct tumor sites within one cancer patient, which cannot be captured by tumor biopsy or excision (section "*Capturing intratumor and intrapatient heterogeneity by ctDNA profiling*"); (iii) ***interpatient tumor heterogeneity***: displays the genetic heterogeneity between patients with the same cancer type (section "*Noninvasive cancer classification and localization*"); (iv) ***temporal tumor heterogeneity***: describes acquired genetic heterogeneity within a patient over time in response to treatment and towards disease progression (section "*Identification of dynamic clonal heterogeneity in response to therapy and towards disease progression by ctDNA profiling*").

2 Technical Aspects of Noninvasive Tumor Genotyping

The success of tumor genotyping from ctDNA requires several technical considerations: First, the analytical sensitivity and specificity of a noninvasive test must be sufficiently high to identify minimal amounts of ctDNA, to detect subtle changes of

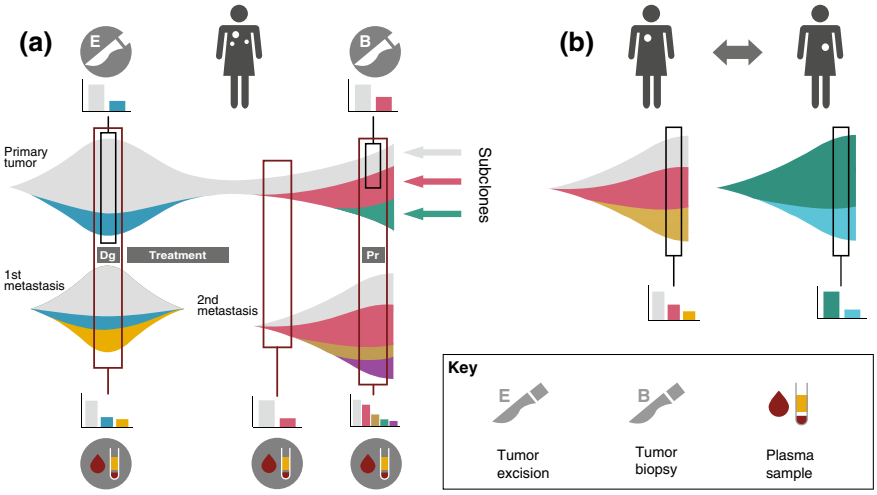

Fig. 1 Different types of tumor heterogeneity identified by tumor and ctDNA genotyping. **a** Schematic illustrating a cancer patient with metastatic disease. Depicted is the evolutionary history of tumor subclones of distinct tumor manifestations at different disease milestones, beginning from cancer diagnosis (Dg) over treatment and surveillance towards disease progression (Pr). During this temporal sequence, both tumor DNA obtained from tumor excisions (E) or biopsies (B) and plasma ctDNA enables the identification and characterization of distinct types of tumor heterogeneity: At diagnosis (Dg), tumor excision (indicated by a scalpel) facilitates the identification of intratumor heterogeneity (left black-bordered bar), while ctDNA analysis (depicted as blood collection tubes) allows assessment of both intratumor and spatial/intrapatient tumor heterogeneity by covering all tumor sites in the patient's body (i.e., genetic landscape of both primary tumor and first metastasis; left red-bordered bar). After completion of treatment, ctDNA genotyping enables the assessment of temporal tumor heterogeneity by detecting minimal amounts of therapy-resistant subclones (gray clone in primary tumor; middle red-bordered bar) and novel emerging subclones during surveillance and towards disease progression (middle and right red-bordered bar). At progression, genotyping of DNA obtained from tumor biopsies (e.g., core needle biopsies) might not fully capture intratumor heterogeneity (right black-bordered bar). Simplified bar plots represent the allelic distribution of genetic alterations characterizing the different subclones captured either by tumor or plasma genotyping. **b** Schematic illustrating interpatient tumor heterogeneity between patients diagnosed with the same cancer type. Differently colored subclones represent the distinct clonal composition of the two tumors at diagnosis and the bar plots represent the allelic distribution of those subclones in each tumor (black-bordered bar)

tumor subclones, and to rule out background noise. Second, the test should cover the entire spectrum of genetic alterations and apply to a wide range of patients to reliably capture all types of tumor heterogeneity (Fig. 1).

Several technologies are available for ctDNA genotyping: polymerase chain reaction (PCR)-based methods that do not rely on sequencing (i.e., quantitative real-time PCR [qPCR], digital PCR [dPCR], and reverse transcription PCR [RT-PCR]) and high-throughput sequencing methods (HTS, next-generation sequencing [NGS], e.g., amplicon-based HTS, targeted hybrid-capture HTS), which allow massive parallel sequencing of DNA molecules in a single flow cell

(Scherer et al. 2017). While PCR-based methods facilitate highly sensitive ctDNA identification down to a detection limit of 0.001–0.0001% allele frequency (AF), they usually cover only one single or a few genetic regions concurrently (Vogelstein and Kinzler 1999; Diehl et al. 2006). Therefore, these technologies represent powerful tools for sensitive detection of stereotypic single genetic aberrations, but are usually unable to fully capture the genetic landscape of most cancer patients (Perdigones and Murtaza 2017; Taly et al. 2013; Waterhouse et al. 2016; Maier et al. 2013; Hehlmann et al. 2014). In contrast, HTS methods can detect the whole spectrum of genomic alterations, including single nucleotide variants (SNVs), insertions/deletions, chromosomal rearrangements, and copy number variants (CNVs). Furthermore, modern HTS assays are usually applicable to a broad population of patients and generally do not require patient-specific optimization, while maintaining high sensitivity for identification of low abundance ctDNA signals (Newman et al. 2014, 2016; Kennedy et al. 2014; Schmitt et al. 2012). Thus, they facilitate comprehensive assessment of intra- and interpatient heterogeneity and allow sensitive detection of clonal evolution over time. However, HTS technologies are not implemented into routine laboratory testing yet; several methodological challenges must be overcome as these methods become routinely used. For example, standardized workflows need to be defined for sample collection and quantification, quality control of sequencing libraries, and bioinformatics analyses including data interpretation (Scherer et al. 2017; Reuter et al. 2015).

Analytical sensitivity of ctDNA detection methods is influenced by several factors. First, sensitivity strongly relies on material quantity and quality. For example, a single gene assay can only achieve a sensitivity of 1 in 20,000 (0.005% AF) if the amount of input cfDNA molecules matches or exceeds this threshold. HTS-based technologies can improve analytical sensitivity below the threshold of single-gene methods, but simultaneous detection of multiple aberrations at the same time is required (Newman et al. 2014; Scherer et al. 2017). The amount of cfDNA molecules recovered by a blood draw can vary substantially, depending on the physiological and pathological condition of a patient. For example, a routine 10 mL blood sample from a healthy individual may yield 4–5 mL of plasma, limiting the available number of analytes to 10,000–20,000 haploid genome equivalents (Perdigones and Murtaza 2017; Devonshire et al. 2014). Levels of cfDNA can be higher in patients suffering from infectious or inflammatory diseases and neoplasms, after myocardial infarction, and during pregnancy (Perdigones and Murtaza 2017).

Second, the concentration of ctDNA in the pool of cfDNA also influences the ability to sensitively detect tumor subclones. Levels of ctDNA can differ significantly between individual patients and highly depend on pathophysiological and technical factors such as disease stage, cancer type, and time point of blood sampling (Perdigones and Murtaza 2017). For example, it has been shown in various cancer entities that pretreatment ctDNA levels in plasma increase with stage and the extent of metastatic spread (Wan et al. 2017; Scherer et al. 2016a; Bettegowda et al. 2014; Parkinson et al. 2016). The same studies demonstrated that patients with certain malignancies including breast and colorectal cancer have significant higher amounts of diagnostic ctDNA than patients with neoplasms confined to the central

nervous system (e.g., glioblastoma) and those with mucinous features (e.g., pancreatic cancer), probably due to physical obstacles such as the blood-brain barrier and mucin preventing ctDNA from entering the circulation (Perdigones and Murtaza 2017; Bettegowda et al. 2014). Furthermore, time delay between blood sampling and plasma processing/storage can cause a relative decrease of ctDNA fractions, because lysis of peripheral blood cells leads to a release of germline cfDNA into the plasma sample. Therefore, timely blood processing and the use of optimized blood collection tubes are recommended to prevent dilution of ctDNA concentrations (Perdigones and Murtaza 2017; Norton et al. 2013; Medina Diaz et al. 2016).

Finally, to achieve sequencing depths that allow sensitive noninvasive ctDNA detection similar to modern PCR-based assays, targeted NGS approaches such as amplicon-based or targeted hybrid-capture HTS are generally preferred over whole genome (WGS) or exome sequencing (WES) technologies, especially when considering current costs and sequencing error rates (Newman et al. 2014, 2016; Narayan et al. 2012; Kinde et al. 2011). While WGS and WES usually achieve sensitivities down to 1%, current targeted HTS methods facilitate ctDNA detection down to a detection limit of 0.001–0.0001% (Newman et al. 2016; Kennedy et al. 2014; Schmitt et al. 2012). However, targeted panels are generally limited to a custom selection of genetic regions and do not comprehensively cover the full genome/exome landscape.

Background DNA errors can be introduced at various stages of ctDNA processing and analysis, limiting specificity and accuracy of PCR- and HTS-based technologies for noninvasive genotyping. This includes errors introduced during PCR amplification, during library preparation, and by oxidative damage during hybridization (Perdigones and Murtaza 2017; Newman et al. 2016). Several in silico error correction and mutation enrichment approaches have been developed in recent years to prevent sequencing data affected by significant background noise, particularly in situations with low ctDNA abundance and when confirmation from paired tumor biopsies is not available (Perdigones and Murtaza 2017; Newman et al. 2016; Kinde et al. 2011; Phallen et al. 2017; Forshew et al. 2012; Li et al. 2008; Thompson et al. 2012).

3 Capturing Intratumor and Intrapatient Heterogeneity by CtDNA Profiling

Tumor tissue genotyping is currently the standard approach for genetic treatment stratification and characterization of tumor genetic landscapes at diagnosis (Fig. 1). However, this approach harbors several limitations: First, the vast majority of tumor tissue genotyping studies focus on one single or a few genetic aberrations in driver genes that have demonstrated prognostic and/or predictive value, ignoring the mutational composition of smaller subclones within the tumor (Robert et al. 2015; Verweij et al. 2004; Maemondo et al. 2010; Misale et al. 2012). Second, one-time

tissue sampling does not allow capturing genetic heterogeneity between distinct and spatially separated tumor sites within a cancer patient (Perdigones and Murtaza 2017; von Bubnoff 2017; Gerlinger et al. 2012). Furthermore, small biopsies of tumor regions limit full genetic characterization of single tumor manifestations (i.e., intratumor heterogeneity, Fig. 1a).

Various recent studies suggested that ctDNA genotyping might have the potential to overcome these shortcomings through its ability to display genetic contributions from all tumor sites, provided that limiting technical factors such as a high rate of background noise can be overcome (Fig. 1a) (Perdigones and Murtaza 2017). Utilizing shotgun massively parallel sequencing, Chang et al. analyzed five distinct tumor tissue samples as wells as a diagnostic plasma sample from a patient with synchronous breast and ovarian cancers. The authors found that the ovarian (four) and breast (one) cancer samples exhibited substantially different patterns of genetic aberrations. Presurgical ctDNA however captured all variants of both cancer types in the plasma and allowed a quantitative estimate of the contributions from each cancer site (Chan et al. 2013). Eight spatially separated tumor biopsy specimens and two serial plasma samples were collected from a patient with ovarian cancer and sequenced by tagged-amplicon deep sequencing (TAm-Seq) in a study conducted by Forshew et al. Two mutations in *EGFR* exon 21 and *TP53* were detected at equally high allele frequencies in ctDNA, sampled 15 and 25 months after initial surgery. Yet, while the same truncal *TP53* variant was identified in all tumor samples at time of debulking surgery, only minute amounts of the *EGFR* mutation were found in two out of eight specimens, suggesting a relative increase and emergence of subclones carrying *EGFR* exon 21 mutation over time (Forshew et al. 2012). In another proof-of-principle analysis, De Mattos-Arruda et al. performed targeted HTS using a 300-gene panel in a patient with metastatic breast cancer and assessed the concordance between genetic aberrations found in the primary tumor, liver metastasis, and serial plasma samples. Diagnostic ctDNA captured all mutations that were either shared between the primary and metastatic tumor sites or unique to both. Furthermore, monitoring of those variants in plasma over time allowed accurate evaluation of tumor response to targeted therapy (De Mattos-Arruda et al. 2014). Murtaza et al. evaluated intratumor heterogeneity in multiple biopsy (obtained at autopsy) and plasma samples collected from a metastatic breast cancer patient over a 3-year clinical course, applying both exome sequencing and targeted amplicon HTS technologies. In this study, the authors found that ctDNA analysis mirrors the genetic hierarchy determined by sequencing of eight distinct tumor specimens (Murtaza et al. 2015). While shared truncal mutations with high allele frequencies could be readily identified in plasma, low abundant private mutations were more difficult to detect, as demonstrated in similar studies (De Mattos-Arruda 2015; Abbosh et al. 2017).

A coupled PCR-NGS approach was used by Jamal-Hanjani et al. to correlate genetic aberrations found in ctDNA with those detected by WES in multiple regions of the same tumor from four patients with early-stage non-small cell lung cancer (NSCLC). 43% of tumor SNVs could also be detected in plasma, including some that were unique to one specific tumor region. However, the probability of detecting

truncal shared mutations was much higher than private SNVs (Jamal-Hanjani et al. 2016). The first systematic comparative sequencing analysis in 100 early-stage NSCLC patients was recently conducted by Abbosh et al. (TRACERx), in which the authors compared the mutational landscapes of ctDNA with those identified by multi-regional phylogenetic profiling of tumor specimens. At least two tumor variants were found in 46 out of 96 diagnostic ctDNA samples (48%). Within the ctDNA-positive cases, almost all (in median 94%) clonal (=shared between all tumor regions) SNVs were detected by ctDNA genotyping, whereas only a minority (in median 27%) of subclonal (=unique to one tumor region) variants could be identified noninvasively. Most interestingly, the success of capturing intratumor heterogeneity in plasma was associated with histological subtype: while ctDNA was detected in the majority of lung squamous cell carcinomas (97%), noninvasive genotyping failed in 81% of patients with lung adenocarcinomas (Abbosh et al. 2017).

Hematologic malignancies, in contrast to solid tumors, are usually circulating diseases; therefore, circulating tumor cells (CTCs) are easily accessible in the blood. Nevertheless, cancer entities such as malignant lymphomas or chronic lymphoblastic leukemia (CLL) often manifest in noncirculating tissue compartments and organs, making CTCs rare and suggesting a potential role of ctDNA profiling for noninvasive tumor genotyping (Scherer et al. 2017). Yeh et al. performed amplicon HTS of 7 genes in patients with CLL and demonstrated that ctDNA reflects both circulating and compartmentalized disease. Moreover, while CTCs could often not be detected at times of overt lymph node (LN) manifestation, ctDNA profiling allowed accurate disease detection and even paralleled the extend of lymphadenopathy in response to therapy over time (Yeh et al. 2017b). Our research group applied CAPP-Seq (Cancer Personalized Profiling by Deep Sequencing), a targeted hybrid-capture HTS technology, to serial paired tumor and plasma samples from a patient who was initially diagnosed with follicular lymphoma (FL), but underwent histological transformation into diffuse large B-cell lymphoma (DLBCL, = transformed FL, tFL). At FL diagnosis, we observed a significant genetic discordance between a left inguinal tumor biopsy and corresponding plasma sample, with 77% of mutations being unique to either tumor or plasma specimens. However, most SNVs genotyped from plasma ctDNA obtained 9 months later (70%) were shared with the patient's retroperitoneal tFL tumor biopsy. Thus, the initial observation suggests that both FL and tFL clones were already present before clinical diagnosis of histological transformation, even if spatially separated (Scherer et al. 2016a).

These studies demonstrate that diagnostic ctDNA profiling probably represents the most accurate and, at the same time, least invasive strategy to capture both intratumor and intrapatient heterogeneity in cancer patients. However, some of the presented literature suggests that technical improvements are needed to increase sensitivity for detection of small subclones harboring minimal amounts of private aberrations.

4 Noninvasive Cancer Classification and Localization

Cancer classification and localization currently relies on (histo-)pathological and radiographic assessment. Yet, accurate evaluation of cell-of-origin (COO) or tissue-of-origin is often hampered by the inability to obtain adequate tissue specimens or in situations in which a primary tumor cannot be identified by conventional diagnostic tools (i.e., cancer of unknown primary, CUP). Molecular features gained from liquid biopsies might help overcome these limitations and support robust cancer classification and tissue-of-origin characterization (Fig. 1b).

Patients with diffuse large B-cell lymphoma (DLBCL) can be stratified using gene expression profiles, allowing prognostic classification into two risk groups (i.e., germinal center B-cell like [GCB] DLBCL, and activated B-cell like [ABC] DLBCL [=non-GCB DLBCL]) based on COO with potential therapeutic implications (Alizadeh et al. 2000; Molina et al. 2014; Wilson et al. 2015; Nowakowski et al. 2015). Current methods for assessment of DLBCL COO either rely on adequate tumor tissue or are limited by low accuracy and reproducibility. Our group demonstrated that DLBCL classification can be robustly performed utilizing genetic information from plasma ctDNA (Scherer et al. 2016a, b). We built a novel classification algorithm based on somatic variants detected by tumor DNA genotyping and assessed its performance on both tumor and plasma samples using CAPP-Seq (Newman et al. 2014; Scherer et al. 2016a; Newman et al. 2016). Tumor COO classification by CAPP-Seq was highly concordant with conventional immunohistochemical classification (i.e., Hans algorithm) and significantly predicted progression-free survival (PFS) (Scherer et al. 2016a; Hans et al. 2004). Most importantly, the concordance rate between tumor and plasma classification was 88%, and DLBCL subtypes predicted solely from plasma were significantly associated with PFS in continuous models (Scherer et al. 2016a).

In a proof-of-principle study ($n = 4$), Fontanilles et al. were able to distinguish brain tumor entities based on the presence or absence of *MYD88* T788C hotspot mutations in ctDNA, using a 32 gene amplicon-based HTS panel (Fontanilles et al. 2017). This variant occurs frequently in primary central nervous system lymphoma (PCNSL) and has not been shown in other primary brain cancer types, including glioblastoma. Therefore, ctDNA genotyping of *MYD88* T788C might have the potential to help identify tissue-of-origin noninvasively in primary brain tumor patients, in whom conventional sampling methods such as needle biopsies are particularly subject to procedural complications (Manoj et al. 2014; Jain et al. 2006; Malone et al. 2015; Ferreira et al. 2006). By integrating three levels of information at diagnosis, Cohen et al. developed a supervised machine learning approach to predict tissue-of-origin in various cancer types. This algorithm took into account ctDNA and protein biomarker levels as well as the gender of patients and was able to localize the source of cancer to two anatomical sites with a median accuracy of 83% (Cohen et al. 2018). Another way to decipher cancer entity and localization noninvasively is by analyzing methylation, nucleosome occupancy patterns, and fragmentation patterns in cfDNA (Wan et al. 2017; Snyder et al. 2016;

Lehmann-Werman et al. 2016; Sun et al. 2015, 2019, Cristiano et al. 2019). Snyder et al. performed ctDNA deep sequencing in five late-stage cancer patients and revealed that nucleosome occupancy patterns allow tissue-of-origin identification and classification of cancers at time of diagnosis (Snyder et al. 2016). Cristiano et al. found that genome-wide fragmentation patterns of cfDNA substantially differ between cancer patients and healthy individuals. Furthermore, genome-wide fragmentation profiles revealed differences associated with specific tissues, enabling tissue-of-origin identification with an accuracy of 61% (Cristiano et al. 2019). In another study, Sun et al. introduced an orientations-aware cfDNA fragmentation analysis that was able to quantify phasing of upstream and downstream fragment ends in tissue-specific open chromatin regions. Using this analysis, the authors were able to distinguish tissue-specific fragmentation patterns of patients with three different cancer types from those of healthy individuals (Sun et al. 2019). Lehmann-Werman et al. and Sun et al. used genome-wide bisulfite sequencing to study methylation profiles of distinct tissue types, facilitating the identification of major tissue contributors to the circulating DNA pool in patients suffering from various pathological conditions, including pancreatic or hepatocellular carcinoma and FL (Lehmann-Werman et al. 2016; Sun et al. 2015).

5 Identification of Dynamic Clonal Heterogeneity in Response to Therapy and Towards Disease Progression by CtDNA Profiling

Repeated biopsies to study clonal evolution over time in response to therapy and towards disease progression are often complicated and invasive, particularly in situations in which tumors are difficult to access (Gerlinger et al. 2012; Murtaza et al. 2013; Shah et al. 2012). Liquid biopsy might represent a superior alternative for dynamic cancer genotyping, because it facilitates (i) the discovery, quantification, and monitoring of emerging genetic processes underlying cancer progression and recurrence, and (ii) the detection of molecular changes that mediate treatment resistance to targeted therapies (Fig. 1a).

Various studies have characterized genomic patterns in plasma over time in response to treatment and towards disease progression. One of the first reports followed 6 patients with advanced breast, ovarian, and lung cancers over 1–2 years by plasma exome sequencing, while they are being treated with conventional and targeted therapies (Murtaza et al. 2013). This study identified treatment-driven selection of subclones harboring resistance-mediating variants in genes like *PIK3CA, RB1,* and *EGFR*, with an increasing relative abundance of subclonal mutations towards radiographic cancer progression (Perdigones and Murtaza 2017; Murtaza et al. 2013). Siravegna et al. exploited serial ctDNA profiling in patients with colorectal cancer to characterize novel resistance processes associated with cetuximab and panitumumab treatment, two antibodies targeting EGFR. They identified genetic aberrations in the plasma of patients with primary or acquired

resistance, including *KRAS, NRAS, ERBB2, MET,* and *EGFR* mutations. Fifty-four percentage of patients harbored one or more aberrations in *RAS* genes, representing the most frequent resistance mechanism to anti-EGFR treatment in CRC (Misale et al. 2012; Siravegna et al. 2015; Diaz et al. 2012). Notably, in three patients who received multiple rounds of EGFR-specific antibody therapy, the authors observed a relative increase of *KRAS*-harboring clones at times of antibody treatment and a decline when antibody therapy was paused. This result indicates that a cancer patient's genome reacts dynamically to intermittent drug schedules and that patients might benefit from pulsatile use of targeted therapies (Siravegna et al. 2015).

Activating mutations in *EGFR* are predictive for clinical response to EGFR tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib in patients with NSCLC. However, resistance to these first-generation TKIs invariably develops within 2 years of treatment. In 60% of patients, resistance is mediated by selection of subclones harboring *EGFR* T790M mutations, which can be detected by ctDNA genotyping with a sensitivity of 70–93% compared to direct tumor genotyping, again highlighting the need for technical improvements to achieve higher detection rates in cases with minute amounts of resistant subclones (Oxnard et al. 2016; Thress et al. 2015a; Chabon et al. 2016b). Third-generation EGFR TKIs (osimertinib and rociletinib) target both activating and T790M resistance mutations. Although having demonstrated activity in T790M-positive NSCLC patients, third-generation agents rarely lead to complete remissions and patients often develop repeated disease progression (Chabon et al. 2016b). Thress et al. and Chabon et al. utilized droplet dPCR and targeted HTS methods to characterize genetic processes in plasma that mediate resistance to osimertinib and rociletinib. While osimertinib resistance was primarily driven by acquired *EGFR* C797S mutations ($\sim$40% of cases), the mechanisms of rociletinib resistance were more heterogeneous and involved *MET, EGFR, PIK3CA, ERRB2, KRAS,* and *RB1* genes (Chabon et al. 2016b; Thress et al. 2015b).

PARP inhibitors (PARP-i) such as olaparib and talazoparib have shown antitumor activity in metastatic prostate cancers with homologous recombination DNA repair defects (e.g., mutations in *BRCA2* or *PALB2*) (Robinson et al. 2015). Goodall et al. and Quigley et al. set out to analyze plasma samples by targeted HTS and WES during PARP-i treatment and at the time of disease progression. Both studies found reversion mutations in *BRCA2* and *PALB2* genes as the major driver of PARP-i resistance, supporting the role of ctDNA profiling as a clinically useful biomarker in this cancer type (Goodall et al. 2017; Quigley et al. 2017).

In patients with myelodysplastic syndrome receiving azacitidine (n = 12), Yeh et al. performed amplicon HTS (243 amplicons) on serial plasma samples throughout the course of treatment and at time of inevitable disease progression. They found several variants emerging before and at the time of clinical progression, including mutations in *NRAS, ASXL1,* and *WT1* (Yeh et al. 2017a). Applying targeted hybrid-capture HTS to serial plasma samples from patients with progressing DLBCL, Rossi et al. (targeting 59 genes) and our group (targeting 268 genes) identified various novel emerging mutations by ctDNA genotyping that are associated with primary therapy-refractoriness or disease recurrence, including variants

in lymphoma driver genes such as *PIM1* and *EZH2* (Scherer et al. 2016a; Rossi et al. 2017). We also utilized our approach for noninvasive genotyping in three patients with progressive disease receiving ibrutinib monotherapy, an inhibitor of B-cell receptor signaling targeting BTK. We identified known emergent resistance mutations in *BTK* that displayed distinct clonal dynamics in two of them. Furthermore, in one case, two adjacent *BTK* mutations encoding the identical amino acid substitution (*BTK* C481S) were found, but they were never observed within the same ctDNA molecule, demonstrating convergent evolution of independent resistant subclones (Scherer et al. 2016a).

Some types of low-grade non Hodgkin lymphomas (NHL) such as FL and CLL can transform into high-grade NHL, most commonly DLBCL. Histologic transformation (HT) is often associated with refractory to treatment and poor prognosis (Montoto and Fitzgibbon 2011; Jain and Keating 2016; Jamroziak et al. 2015; Kridel et al. 2017). Sequencing analysis of tumor specimens biopsied at HT diagnosis revealed several genes frequently mutated in transformed patients, including *TP53, MYC, CCND3,* and *FOXO1* (Kridel et al. 2017; Yano et al. 1992; Lo Coco et al. 1993; Rossi et al. 2011). Early identification and treatment of patients undergoing HT is important; however, there are currently no strategies available that enable early detection of genetic changes that might predict a transformation event (Yeh et al. 2017b). Yeh et al. performed low-coverage WGS and WES on plasma in three CLL patients at initial diagnosis and transformation (Richter's syndrome, RS). Several new emerging HT-specific CNVs and SNVs, including 17p deletion, *TP53*and *SF3B1*mutations, could be identified at the time of transformation by noninvasive genotyping; moreover, the HT-specific alterations could even be assigned to the "compartment of origin" (e.g., LN or bone marrow) (Yeh et al. 2017b). Our group applied CAPP-Seq to tumor samples taken at initial FL diagnosis and plasma samples collected at the time of either HT (transformed FL, tFL) or FL progression without transformation (non-transformed FL, ntFL) to conduct comparative genomic analyses over time. We observed a greater evolutionary distance among tumor-plasma pairs associated with transformation, with a significantly higher fraction of emergent variants in ctDNA distinguishing tFL from ntFL patients. This result suggests that the extent of clonal divergence in plasma might be a potential biomarker for HT prediction (Scherer et al. 2016).

6 Conclusions

Every aspect of cancer research and the way patients with malignancies are treated is and will be influenced by tumor heterogeneity (Alizadeh et al. 2015). Published studies presented in this chapter suggest a major future role of ctDNA genotyping for identification and characterization of different tumor heterogeneity types, with several important advantages compared to direct tumor genotyping. Due to recent technical improvements, in particular HTS technologies, we foresee that noninvasive assessment of tumor heterogeneity will soon be shown to have clinical utility

for accurate classification of patients risk groups, for identification of patients most likely to benefit from targeted and personalized therapeutic strategies, and for assessment of genetic factors driving treatment resistance and disease progression. Ultimately, future clinical trials that incorporate ctDNA profiling for serial genotyping have to demonstrate improved clinical outcomes.

However, several aspects of this approach remain subjects of active investigation and researchers just started to understand how we interpret and use information gained from ctDNA genotyping to advance our understanding of cancer and to achieve better clinical results. For example, it is still unclear whether discordant genotyping results from plasma and tumor biopsies should be interpreted as presence of intrapatient (or spatial) tumor heterogeneity or as false positive calls due to background noise. Additionally, some studies show that detection rates of certain genetic aberrations in plasma are relatively low compared to tissue genotyping (e.g., 70–93% detection rate of *EGFR* T790M), highlighting the need for further improvements of sensitivity to identify small tumor subclones. Finally, biological factors including vascularization and physical obstacles (e.g., blood–brain barrier) influence the way ctDNA enters circulation; thus, it is not clear whether all tumor manifestations contribute equally to ctDNA genetic patterns and how this affects correct quantification of tumor subclones.

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Review ctDNA and Breast Cancer

Florian Clatot

Breast cancer (BC) is the most frequent cancer among women with 1.7 million cases each year worldwide, and the leading cause of cancer death in women with 522,000 deaths in 2012 (Ginsburg et al. 2016). Only 10% of BC are diagnosed at a metastatic stage, but around 20–30% of the early breast cancer (EBC) will present a metastatic relapse during follow-up. Routine clinical and histological prognostic factors such as tumor grade, tumor stage or lymph node involvement are established at the beginning of BC management. These markers are mandatory to define the therapeutic strategy. Sometimes, additional biopsies are performed during BC management in case of accessible metastases. But physicians are looking for dynamic non-invasive tools able to account for the global cancer behavior. CA 15-3 assessment is a circulating biomarker that is correlated with BC burden. Yet, lack of sensitivity and specificity of CA 15-3 limits its use to some metastatic breast cancer patients (Duffy et al. 2010). Furthermore, BC is a highly heterogeneous disease with a clinical evolution influenced by molecular modifications that happen under treatment exposure. Being able to monitor these modifications and adapt treatments using non-invasive markers has been a long quest that is nearing completion. Decades ago, the proof of principle that tumor-associated DNA could be detected in blood samples was made in BC with the detection of increased levels of cell-free DNA (cfDNA) (Leon et al. 1977). Indeed, since cfDNA reflects the amount of DNA released by apoptotic and necrotic cells (either tumoral or not tumoral) (Diaz and Bardelli 2014), assessing the global cfDNA amount during cancer evolution

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was supposed to be related to cancer evolution. This approach that does not need a cancer-specific target was further improved by assessing circulating tumor DNA (ctDNA) using cancer-specific DNA alterations. In this review, we will discuss the potential clinical interests and limitations of the cf/ctDNA approach in BC at the different times of BC management, with a particular emphasis on recent data from clinical trials. Of note, pathophysiology of ctDNA release into the circulation, technical aspects of ctDNA detection and analysis, but also circulating tumor cells in BC have been detailed in dedicated chapters of this book and will not be discussed here.

1 cfDNA and ctDNA Use at Screening and Diagnosis

The simplest way to measure cfDNA is global quantification, which indeed has been shown to increase among patients with BC compared to benign breast disease or healthy controls (Huang et al. 2006). Furthermore, global cfDNA level has also been related to cancer stage, with a higher rate of cfDNA measured in case of tumors with advanced stages (Tangvarasittichai et al. 2015). Other approaches based on global DNA modification in case of cancer, such as loss of heterozygosity (LOH), microsatellite instability (MI) or hyper-methylation (Shaw et al. 2000; Silva et al. 2016) and *ALU* DNA integrity (Umetani et al. 2006) were also investigated but lacked of sensitivity. More recently, based on ctDNA analysis, a prospective study of *PIK3CA* circulating mutation detection before and after surgery in EBC showed that 14 of the 15 patients with mutation in primary tumor had a concordant mutation detectable in blood (Beaver et al. 2014). Yet, *PIK3CA* mutation only occurs in 30–50% of BC cases and its detection can not be used alone as screening test. More recently, the use of more complex molecular tools such as next-generation sequencing (NGS) or deep sequencing able to look for cancer-specific mutations in several genes, have also been tested in the screening setting, with a higher rate of detection (Kirkizlar et al. 2015).

Talking about a diagnostic tool implies to evaluate the performance of such tool. A recent meta-analysis reported a sensitivity of 0.7 and a specificity of 0.87 for the use of cf/ctDNA as diagnostic tool among 24 studies. When considering only the 6 studies using qualitative modern ctDNA evaluation, screening performance was improved with a sensitivity of 0.88 and a specificity of 0.98 and could compare favorably with digital mammography (Lin et al. 2017). Of note, qualitative ctDNA analyses imply a high cost and a level of technicity that are to date hardly compatible with a screening tool. Furthermore, these 6 studies only included 126 cases and 190 controls and some of the studies were limited to early BC patients (Kirkizlar et al. 2015) while others concerned metastatic BC patients (Dawson et al. 2013). In the same line, a recent analysis of 32 patients with stage I or II BC by massively parallel sequencing identified somatic mutations in 19 cases (59%) (Phallen et al. 2017). Knowing that ctDNA amount is much more higher among metastatic BC patients compared to early BC patients and vary greatly even

between patients with the same stage of disease (Bettegowda et al. 2014), the sensitivity and specificity of ctDNA as a screening tool in BC should be assessed in large prospective studies and homogeneous populations. Of note, technological improvements in coming years (such as plasmapheresis, ctDNA capture using implanted devices or selection of DNA fragment sizes between 90 and 150 base pair before sequencing) may overcome the low amount of ctDNA currently available in BC early stage, and thus improve sensitivity (Mouliere et al. 2018; Wan et al. 2017).

2 ctDNA Use for Early Breast Cancer Management

2.1 Early BC Classification

Early BC is a heterogeneous disease both at the molecular and clinical levels. Daily practice is often based on a classification of breast cancers assessed by immune-histochemical expression of ER and PgR receptors (Hormone receptors, HR), HER2 receptor, and Ki67.

Luminal BC are characterized by the expression of ER/PgR and count for 70% of diagnosed breast cancers. These cancers are associated with a better prognosis, even if risk of relapse extends commonly over 10 years from diagnosis. Among luminal cancers, a distinction is made between tumors with a low proliferation rate (assessed by Ki67) and referred as luminal A, and tumors with a high proliferation rate and/or a *Her2* amplification, referred as luminal B (Senkus et al. 2015). Luminal A cancers present frequent *PIK3CA* mutation (49% of the cases) (Cancer Genome Atlas Network 2012) while luminal B present a high rate of *PIK3CA* mutation (29%), as well as a high rate of *TP53*mutation (29%). Besides luminal cancers, the HER2 enriched group represents 15% of the BC spectrum. This group is characterized by an amplification of the *HER2* gene and no HR expression, and is associated with a more aggressive phenotype. HER2-enriched tumors, present a high rate of *TP53* mutations (75%) and *PIK3CA* mutations (42%). Interestingly, if *HER2* amplification is a strong factor of poor prognosis, it is also a strong predictive factor of improved outcome under targeted anti-HER2 treatment, such as trastuzumab (Joensuu et al. 2009). Finally, the last 15% BC belongs to the triple-negative breast cancer group (TNBC). This group shares the lack of expression of ER, PgR and HER2 but also a high *TP53* mutation rate of 84%, a *PIK3CA* mutation rate of only 7% but a *PTEN* loss/mutation in 35% of the cases (Cancer Genome Atlas Network 2012). TNBC is a heterogeneous group, overall associated with a poor prognosis with usually highly proliferative tumors and relapses mainly observed during the 3 first years of follow-up.

Overall, if there is no unique detectable DNA abnormality in early BC- such as the exon 12*K-RAS* mutations observed in around 70% of pancreas adenocarcinomas (Boeck et al. 2013)- a driver DNA alteration can be identified at an individual level in 95% of the cases (Nik-Zainal et al. 2016), providing a target to monitor in

ctDNA. Interestingly, *PIK3CA* mutations are mostly recurrent (E542K, E545K and H1047R representing 80% of the whole *PIK3CA* mutations) (Guerrero-Zotano et al. 2016). Thus, few digital PCR (dPCR) assays can reliably investigate the presence of a circulating *PIK3CA* mutation among numerous BC patients at low cost. In contrast, *TP53* mutations and most of the other DNA alterations found in EBC tumors are not recurrent, and warrants either a first step of primary tumor sequencing before setting up a dedicated dPCR assay, or a deep ctDNA sequencing.

2.2 cf/ctDNA Use as a Prognostic Marker in EBC

Total quantification of cfDNA has been associated with a poor overall survival. Fujita et al. analyzed retrospectively the overall survival (OS) of 336 stage I/II early BC. In multivariate analysis, patients with high amount of cfDNA (upper tertile) had a poorer disease-free survival (DFS) (HR = 2.7 [1.5–4.9], $p = 0.001$) and OS (HR = 4.0 [1.6–10.1], $p = 0.003$) compared to patients with intermediate or low amount of cfDNA (Fujita et al. 2012). The same team reported comparable results in the neoadjuvant setting (Fujita et al. 2014). Oshiro et al. investigated the prognostic value of ctDNA at diagnosis in 313 stage I–III early BC. First, they looked for *PIK3CA* mutations in tumor biopsies and found 110 (35%) positive cases. Then, they assessed the presence of a matched circulating *PIK3CA* mutation by dPCR, which was found in 25 plasma samples. Among these 25 patients, patients with a high (above the median) amount of ctDNA were associated with a worse DFS and OS compared to the others (Oshiro et al. 2015). But the very low number of patients concerned (4%) prevent from any definitive conclusion. Furthermore, Garcia-Murillas et al. did not find any prognostic value in DFS in case of ctDNA detection at diagnosis ($n = 29$) compared to no detection ($n = 13$) in a cohort of early BC (Garcia-Murillas et al. 2015).

2.3 ctDNA, Tumor Heterogeneity and Targetable DNA Alterations

Intratumor heterogeneity (ITH) is one potential issue when dealing with EBC. Indeed, usual prognostic or predictive factors may be misestimated between initial biopsy and whole surgical specimen, in particular for large tumors (Petrau et al. 2015). This point is of particular interest since neoadjuvant treatments in EBC are decided based on conventional biopsies. When considering DNA alterations, liquid biopsies may theoretically overcome ITH. ITH at a genomic level in EBC has been investigated by Yates et al. who performed a NGS analysis of 8 biopsies in primary tumor of 12 EBC patients (Yates et al. 2015). For each of the BC investigated, at least one clonal somatic driver mutation or copy number event was shared by all samples. On the other hand, among the 12 EBC investigated, 8 demonstrated statistically significant spatial heterogeneity of point mutations. Finally, in 4 of the

12 cancers, a subclonal driver mutation could be observed in some but not all of the biopsies investigated. Comparable results have been reported by Desmedt et al. who performed a targeted analysis of 171 samples from 36 multifocal primary tumors and identified an inter-region heterogeneity in oncogenic mutations in 12 (33%) of the cases (Desmedt et al. 2015). Thus, depending on the location of the biopsy, a subclonal driver mutation may be found, or not. Very few studies have investigated the potential interest of ctDNA analyses in overcoming ITH. Yet, Murtaza et al. showed that all the metastatic-clade mutations that were initially present in one or 2 of the primary tumors biopsies could be detected in plasma samples (Murtaza et al. 2015).

2.4 ctDNA Use as a Tool for Monitoring Cancer Dynamic

The potential interest of ctDNA in monitoring minimal residual disease has been reported by Garcia-Murillas et al. in a prospective study published in 2015. Fifty-five early BC patients treated by neoadjuvant chemotherapy were included. First, a targeted massively parallel sequencing (MPS) of primary tumor biopsy investigated 14 BC driver genes mutations and identified at least one mutation in 43 of the 55 cases (78%). Using a dedicated dPCR assay, corresponding mutation was then found in ctDNA in 70% of the cases at baseline. Interestingly, while baseline ctDNA amount was not related to DFS, the persistence of a detectable mutation in ctDNA 2–4 weeks after surgery (7 cases among 37 patients) was associated with a very high risk of early relapse (HR 25.1 CI95[4–130]). Moreover, using repeated sampling during follow-up, ctDNA mutation could be detected for 12 of the 15 patients that experienced relapse, compared to 1 of the 28 patients that did not relapse. Finally, detection of mutation in ctDNA had a median lead time of 7.9 months over clinical relapse. Olsson et al. reported highly comparable results in a retrospective study of 20 early BC (Olsson et al. 2015). More recently, Riva et al. took advantage of the very high amount of *TP53* mutations in triple-negative breast cancer. A deep sequencing of primary tumor samples identified a *TP53* mutation in 40 of the 46 early TNBC patients (87%) included prospectively. Corresponding *TP53* mutation was found in ctDNA at baseline by droplet dPCR (ddPCR) for 27 of the 36 patients (75%) evaluable. Then, all the patients underwent preoperative chemotherapy. Interestingly, while cfDNA increased during the first cycles in relation to DNA release by necrotic tumor cells, ctDNA decreased in the mean-time. Persistence of a detectable ctDNA after the first cycle of chemotherapy was associated with a poor outcome both in DFS and OS (Riva et al. 2017). Finally, Chen et al. assessed the prognostic value of the detection of a circulating tumoral mutation at the end of neoadjuvant chemotherapy among 38 triple-negative early BC. All the 4 patients with a detectable mutation at the end of neoadjuvant chemotherapy relapsed (100% specificity) while 9 other patients without circulating mutation relapsed during follow-up (31% sensitivity) (Chen et al. 2017). Taken together, the results of these studies suggest that such non-invasive approach could

identify patients with very high risk of recurrence. Next step will be to improve outcome of patients with ctDNA persistence/relapse by proposing dedicated treatments.

3 ctDNA Use for Advanced Breast Cancer Management

3.1 cf/ctDNA and Prognostic Value in Advanced Breast Cancer

cfDNA amount (Cheng et al. 2018; Clatot et al. 2016) as well as ctDNA amount have been correlated to overall survival (Bettegowda et al. 2014; Dawson et al. 2013).

3.2 ctDNA, Tumor Heterogeneity and Targetable DNA Alterations

Beyond ITH, another level of complexity among heterogeneity arises when considering the natural history of breast cancer, i.e., development of metastases, or anti-cancer treatment influence (Juric et al. 2015). In a proof-of-concept study, Murtaza et al. reported a high concordance between exome sequencing of ctDNA and matched tumor biopsy sequencing both for mutation detection and copy number alteration (CNA), even if CNA evaluation was highly influenced by the mutant allele fraction in plasma samples (Murtaza et al. 2013). In another study, the same team investigated the correspondence between genomic alterations in serial ctDNA analyses ($n = 9$) compared to primary ($n = 3$) and metastases biopsies ($n = 5$) in a HR + HER2 amplified metastatic BC patient. Interestingly, they reported that mutations observed ubiquitously in all tumor samples as well as metastatic-clade mutations could be identified in ctDNA. Furthermore, a *PIK3CA*-E542K hotspot mutation hardly detectable in 2 tumor biopsies was reliably identified in ctDNA during disease progression, before disappearing with a treatment change (Murtaza et al. 2015). This observation, also reported by other teams (Butler et al. 2015; De Mattos-Arruda et al. 2014), underlines the potential interest of single blood sample analysis in identifying targetable alterations that could emerged in any of the disseminated metastases. Recently, data from the BELLE-2 trial showed that the concordance between tumor *PIK3CA* mutation and matched ctDNA mutation detection was 77% (342/446). Interestingly, among the 307 patients without *PIK3CA* mutation in tumor tissue, 64 (21%) had a detectable circulating *PIK3CA* mutation, probably because of tumor changes during the interval between the biopsy for initial diagnostic and subsequent blood analysis after multiple lines of treatment (Baselga et al. 2017).

Due to discordances between primary tumor and metastases in HR and HER expression (Amir et al. 2012; Thompson et al. 2010), confirmatory tissue sampling

may be valuable for determining an endocrine or HER2 targeted therapy. Nevertheless, such biopsy is not always feasible. In that context, HER2 amplified copy number determination in plasma cfDNA has been shown to be an interesting tool with positive and negative predictive values of 70% and 92%, respectively (Gevensleben et al. 2013).

3.3 ctDNA and Drug Resistance in Advanced Breast Cancer

3.3.1 Unspecified Intrinsic Subtype

At a glance, many drugs or targeted treatments can be efficient in the metastatic setting, but resistance either primary or after few weeks/months is the rule. To date, clinical progressions are mostly diagnosed on CT-scan evaluations. The potential clinical interest of ctDNA use in advanced breast cancer was highlighted in the landmark study published by Dawson et al. in 2013. In this study, targeted or whole-genome sequencing was performed in tumor biopsies from 52 metastatic BC patients. For the 30 patients with genomic alterations suitable for monitoring, serial plasma analyses were performed using either dPCR assays or direct plasma deep sequencing. Besides ctDNA analyses, CTC counts and CA 15-3 measurements were performed during a 2-years follow-up. Overall, ctDNA was detectable in 29 of the 30 patients assessed (97%), at least one CTC was detectable for 26/30 patients (87%), and CA 15-3 showed elevated values for 21/27 patients (78%). Furthermore, if all the 3 biomarkers variations were related to treatment responses seen on imaging, ctDNA rates showed the greater dynamic range variations and greater correlation with changes in tumor burden (Dawson et al. 2013).

Besides genomics alterations, epigenetic modifications frequently occur in tumors cells. Evaluation of the DNA methylation status can also be performed in ctDNA (see the review (Schwarzenbach and Pantel 2015). Recently, the prospective validation of the prognostic value of a methylation index based on 6-genes (*AKR1B1*, *OXB4*, *RASGRF2*, *RASSF1*, *HIST1H3C*, and *TM6SF1*) showed that among the 141 metastatic BC patients assessed, those with a low methylation index after 4 weeks of treatment had better PFS and OS outcomes compared to the patients with a high methylation index. The prognostic value of this biomarker remained significant in a multivariate analysis that included a CTC evaluation (Visvanathan et al. 2017).

3.3.2 HER2 Amplified Breast Cancer

PI3K Pathway

The PI3K/AKT/mTOR pathway is very frequently altered in BC both in EBC (Cancer Genome Atlas Network 2012) and metastatic BC (Arnedos et al. 2015). Regulation of the PI3K pathway and potential deregulations of this pathway in cancer are complex (Guerrero-Zotano et al. 2016). Basically, PI3K is a heterodimer consisting of a p85 regulatory subunit and a p110 catalytic subunit. PI3K is activated by growth factor receptors such as the HER family (including EGFR and

HER2), IGFR or FGFR. Activated PI3K then activates AKT which in turns activates mTOR1 and promotes cell proliferation and tumor growth. PTEN inhibits PI3K. Most of the *PIK3CA* mutations are recurrent [E542K, E545K and H1047R mutations explain around 80% of all the *PIK3CA* mutations observed (Hortobagyi et al. 2016)] making *PIK3CA* mutation detection in ctDNA by dPCR easy to proceed, even if a complete evaluation of the PI3K pathway activation (including *PTEN* mutation or deletion or *AKT* activating mutation (Hortobagyi et al. 2016) makes more biological sense. Of note, PI3K pathway activation seems of peculiar importance in endocrine or HER2-targeted therapies resistance (Guerrero-Zotano et al. 2016; Ma et al. 2015). The diagnostic accuracy of *PIK3CA* mutation status by cfDNA analysis has been recently estimated in a meta-analysis. This meta-analysis was limited to 247 patients included in six studies and pooled data from early BC and metastatic BC. Overall, a sensibility of 0.86 (95%CI [0.32–0.99]) and specificity of 0.98 (95%CI [0.86–1.00]) was found, making the cfDNA analysis a reliable tool in determining the *PI3KCA* mutation status (Zhou et al. 2016).

Several retrospective analysis of large HER2-amplified metastatic BC neoadjuvant trials have highlighted a worse pathological complete response (pCR) in case of PI3Kpathway activation by *PTEN* loss and/or *PIK3CA* mutation (Loibl et al. 2014; Majewski et al. 2015). In the same line, the biomarker analysis of the CLEOPATRA trial which tested the addition of pertuzumab to a docetaxel + trastuzumab combination in first-line HER2 + MBC patients found a worse outcome in PFS in case of *PIK3CA* mutation (Baselga et al. 2014). The combined analysis of the BOLERO-1 and BOLERO-3 trials which investigated the benefit of addition of everolimus to trastuzumab + chemotherapy in advanced HER2 + BC also found a worse PFS outcome in case of *PIK3CA* mutation or other activation of the PI3K pathway, such as *PTEN* loss (André et al. 2016). Surprisingly, these homogeneous results seen in the neoadjuvant and metastatic settings are not observed in the adjuvant setting, since some retrospective analysis of prospective trials found a worse outcome in case of *PIK3CA* mutation/*PTEN* loss (Jensen et al. 2012), while others did not (Perez et al. 2013; Pogue-Geile et al. 2015). Of note, all these trials were performed on tumor samples but not in ctDNA. It seems of particular interest to evaluate to prognostic and predictive values of *PIK3CA* circulating mutations for HER2 + patients.

HER2 Amplifications

The potential interest of ctDNA in detecting HER2-targeted treatment resistance has been investigated in 52 plasma samples from 18 metastatic BC patients with HER2-amplified tumors. These patients were included in a prospective trial evaluating the efficacy of pyrotinib, a HER2 inhibitor. HER2 copy number variation (CNV) in plasma samples was found to be related to outcome with a progression of the disease in case of HER2-CNV increase. Of note, this result has to be considered regarding the small number of patients included. Furthermore, 5 out of the 18 patients included (28%) had no circulating HER2 amplification detectable, whatever the time point, which undermines the sensitivity of such marker of resistance (Ma et al. 2016).

3.3.3 Hormone Receptor Positive Breast Cancers

HER2 Mutations

HER2 targeted therapies usually do not benefit patients without HER2 amplification. But some recurrent *HER2* mutations have been recently identified (Bose et al. 2013) among *HER2* nonamplified patients. A recent review based on 12,905 BC cases reported a mutational rate of 2.7% (Petrelli et al. 2017). Since these mutations are activating ones, a potential benefit of HER2 targeting treatment- in particular the pan-HER2 inhibitor neratinib- was expected in that setting. Indeed, a dedicated phase II trial reported that an *HER2* mutation was found in 9 of 381 HER2 non-amplified BC patients (2.4%), with a higher rate of detection among lobular carcinoma (7.8%). The primary endpoint was the clinical benefit rate (CBR, including partial or complete responses, as well as stable disease for at least 24 months). Finally, 16 patients harboring a *HER2* mutation who had received a median of 3 metastatic regimens before inclusion were treated by neratinib. The CBR was 31%, which met the primary endpoint, and the PFS was 16 months. Interestingly, 14 of the 16 patients had plasma samples available at baseline, and 12 out of these 14 patients (86%) had a detectable *HER2* mutation in ctDNA. Furthermore, the evaluation of archival plasma from 1584 HER2 non-amplified BC patients confirmed a rate of circulating HER2-mutation detection of 3% (Ma et al. 2017). Despite the small number of patients treated in that study, and even if HER2-mutation is rare in BC, the use of ctDNA might help screening which patients harbor a *HER2* mutation and could benefit from neratinib.

PI3K Pathway

The PALOMA-3 randomized trial compared fulvestrant + palbociclib versus fulvestrant + placebo in HR + MBC patients who progressed on previous hormone therapy. A sub-analysis investigated the prognostic and predictive values of circulating *PIK3CA* mutation detection among 395 of the 521 included in the PALOMA-3 trial. Four mutations were investigated. One-third of the patients investigated had a detectable circulating *PIK3CA* mutation at baseline, which was not related to a better outcome in terms of DFS and was not predictive of a differential outcome between the two treatment arms investigated (Cristofanilli et al. 2016). On the other hand, the same team analyzed 73 patients with matched plasmas at baseline and day 15 of treatment. They reported a better outcome among patients below median value of circulating *PIK3CA* mutation at day 15 compared to patients above (HR 3.94 95CI[1.61–9.64], $p = 0.0013$). Thus, such mutation variation may be an early surrogate marker of outcome (O'Leary et al. 2018a).

Lack of circulating *PIK3CA* mutation prognostic or predictive values at baseline was also observed in the retrospective analysis of 550 patients included in the BOLERO-2 trial, which randomized everolimus (mTOR inhibitor) + exemestane versus placebo + exemestane in HR + MBC patients progressing on AI treatment (Moynahan et al. 2017). Recently, the BELLE-2 trial also reported an analysis of the clinical value of circulating *PIK3CA* mutations. This trial included 1147 h + MBC patients resistant to AI who were randomized to receive oral buparlisib

(pan-PI3K inhibitor) + fulvestrant or placebo + fulvestrant. Among the 387 patients without *PIK3CA* circulating mutation, no difference in PFS was observed according to the 2 treatment arms. In contrast, among the 200 patients with a *PIK3CA* circulating mutation, those receiving buparlisib had a much better outcome in terms of PFS than those in the placebo arm (HR 0.58, 95CI[0.41–0.82]). Interestingly, when considering patients with a "PI3K pathway activated" defined as a PIK3CA activating mutation in tumor tissue or a lack of PTEN expression, these patients had the same outcome compared to the whole population. Thus, this trial suggests that circulating *PIK3CA* mutation but not PI3K status in tumor tissue could be a predictive marker of response to buparlisib (Baselga et al. 2017).

Taken together, these analyses of large randomized trials suggest that the *PIK3CA* circulating mutation prognostic and predictive values may vary according to the peculiar drug investigated. Thus, some recent clinical trials investigating a PI3K-pathway inhibition in HR + MBC patients, such as the SANDPIPER phase III trial (NCT02340221), will be restricted to patients harboring a *PI3KCA* mutation.

ESR1 Mutations

Circulating *ESR1* mutation assessment will doubtless be the first application of ctDNA in daily practice for BC management. The importance of point-mutation in estrogen receptor (ER) was first suggested from E380Q and Y537S directed mutations in breast cancer cell lines (Pakdel et al. 1993; Weis et al. 1996). The first observation of *ESR1* activating mutation was found by sequencing in a BC metastasis in 1997 (Zhang et al. 1997). Yet, due to the low incidence of *ESR1* mutations in primary BC tumors (0–2%) (Cancer Genome Atlas Network 2012; Toy et al. 2013), their potential importance was first underestimated. In contrast, the analysis of BC metastases after exposure to endocrine therapies showed that tumor resistance was related to few *ESR1* mutations which modify the receptor conformation and provide an ER auto-activation (Merenbakh-Lamin et al. 2013; Robinson et al. 2013; Toy et al. 2013). Since *ESR1* mutations occur under hormone therapy, the liquid biopsy approach is of particular interest by allowing regular assessment of the *ESR1* mutation status during treatment. The proof of concept of *ESR1* mutation detection in plasma samples was provided in 2015, with a mutation detected in 9 out of 48 h + MBC patients (Guttery et al. 2015). A good overall correlation between plasma samples and matched biopsies of metastases was then observed with a sensitivity of 75% and a specificity of 100% (Sefrioui et al. 2015) (Schiavon et al. 2015). Deep sequencing of metastases after tamoxifen or AI exposure may detect various *ESR1* mutations, with many of them being subclonal (Lefebvre et al. 2016; Magnani et al. 2017). In contrast, circulating *ESR1* mutations are clearly associated with previous AI exposure (Schiavon et al. 2015; Spoerke et al. 2016; Yanagawa et al. 2017) and are unusual after tamoxifen-only exposure (Fribbens et al. 2016).

Despite lack of standardization between detection methods, global overall incidence of circulating *ESR1* mutations after progression on AI is around 30%. Interestingly, 5 mutations (E380Q, D538G, Y537S/N/C) explain more than 80% of

all the *ESR1* circulating mutations observed. Of note, *ESR1* circulating mutations are polyclonal in 30–50% of the cases (Chandarlapaty et al. 2016; Clatot et al. 2016; Fribbens et al. 2016; Spoerke et al. 2016).

During breast cancer management, the main AI exposure occurs in the adjuvant setting, but the incidence of *ESR1* circulating mutation at the end of hormonal therapy adjuvant treatment remains poorly investigated. Only one retrospective study of 39 h + MBC patients that experienced relapse at least 6 months after the end of their AI-based adjuvant treatment reported that none of the patients had a detectable circulating mutation at the end of the adjuvant treatment. In contrast, 2/35 of these patients had a circulating mutation at time of metastatic relapse (Allouchery et al. 2018). These results are in line with the low incidence of *ESR1* circulating mutations observed in two cohorts of patients only exposed to AI during the adjuvant treatment with 2/75 (Schiavon et al. 2015) and 11/97 (Chandarlapaty et al. 2016) positive cases at time of metastatic relapse. Taken together, these results suggest that a daily use of *ESR1* circulating mutation assessment should be restricted to the metastatic setting.

Two studies have assessed the prognostic value of a circulating *ESR1* mutation on overall survival (OS). The first study was a retrospective analysis of plasma samples from 541 of the 742 h + MBC patients included in the BOLERO-2 trial. These patients had at least progressed on AI (letrozole or anastrozole) and were randomized between the everolimus + exemestane combination versus placebo + exemestane. Two circulating mutations have been assessed by ddPCR (D538G and Y537S). While median OS was 32 months for wild-type patients, a significant decrease in OS was observed among the 156 patients (28.8%) harboring an *ESR1* circulating mutation: 26 months in case of D538G mutation ($n = 83$, $p = 0.03$), 20 months in case of Y537S mutation ($n = 42$, $p = 0.003$) and even 15 months in case of dual mutation ($n = 30$, $p < 0.001$). This poor OS in case of circulating *ESR1* mutation was confirmed by multivariate analysis (HR = 1.6 [1.26–2.00], $p < 0.001$) (Chandarlapaty et al. 2016). The second study included retrospectively 141 h + MBC on progression after a first line of AI in the metastatic setting. Four mutations were investigated (D538G, Y537S/N/C). The 15.5 months OS was significantly worse among the 43 patients (30.6%) with a circulating *ESR1* mutations compared to the 24 months OS of patients without *ESR1* mutation ($p = 0.0006$). This poor impact on OS was confirmed by multivariate analysis (HR = 1.9 [1.3–3.0], $p = 0.002$) (Clatot et al. 2016).

Besides its prognostic value, the predictive value of *ESR1* circulating mutation has also been investigated. A retrospective analysis from the SoFEA trial evaluated the impact on PFS of a circulating *ESR1* mutation among patients randomized between exemestane or fulvestrant (± anastrozole) after progression on AI. Plasma samples from 161 of the 723 patients (22.4%) included in the SoFEA trial were analyzed. Under exemestane exposure, presence of an *ESR1* circulating mutation was associated with a shorter PFS compared to patients without *ESR1* circulating mutation (PFS 2.6 months vs. 8 months, respectively, HR = 2.12 [1.18–3.81]) (Fribbens et al. 2016). Comparable results have been reported elsewhere (Schiavon et al. 2015). Yet, in the retrospective analysis of the BOLERO-2 trial, treatment by

exemestane was only associated with a non-significant trend over shorter PFS (2.8 months in case of mutation vs. 3.9 months without mutation, $p = 0.16$) (Chandarlapaty et al. 2016). Concerning fulvestrant exposure, retrospective analyses of parts of the patients included in the SoFEA and FERGI trials reported a comparable PFS among patients with or without circulating *ESR1* mutation (Fribbens et al. 2016; Krop et al. 2016). Data from the PALOMA-3 trial, which compared a fulvestrant + palbociclib combination versus fulvestrant + placebo showed a shorter 3.6 months PFS among the 28 patients with a circulating *ESR1* mutation treated by fulvestrant + placebo compared to 5.4 months PFS among the 92 patients without circulating *ESR1* mutation receiving the same regimen (*p*-value nonreported) (Fribbens et al. 2016). Concerning the exemestane + everolimus combination, the large retrospective study from the BOLERO-2 trial assessed the impact of 2 circulating *ESR1* mutations (D538G and Y537S) on treatment outcome. Patients harboring a D538G mutation had a better PFS under an exemestane + everolimus combination compared to exemestane + placebo (5.8 and 2.7 months, respectively, $p < 0.001$). Yet, among patients receiving an exemestane + everolimus combination, D538G circulating mutation was associated with a worse prognostic compared to patients without mutation (5.8 vs. 8.5 months PFS, respectively). In contrast, and even if the limited number of patients analyzed prevent from any definitive conclusion, no benefit of the everolimus + exemestane combination was observed over exemestane + placebo in case of circulating Y537S mutation ($n = 42$) (Chandarlapaty et al. 2016).

Finally, the retrospective analysis of plasma samples from 360 of the 521 patients (69%) included in the PALOMA-3 trial reported a comparable outcome under fulvestrant + palbociclib for patients with ($n = 63$, PFS 9.4 months) or without mutation ($n = 177$, PFS 9.5 months) (Fribbens et al. 2016).

Besides data from the BOLERO-2 trial, there is no clinical evidence for differential prognostic values regarding a peculiar *ESR1* mutation, even if published studies would have lacked power for detecting such differences. Yet, preclinical data have recently reported the functional behavior of the most frequent *ESR1* mutations using HR + BC cell lines (MCF7) as well as xenograft. Interestingly, the tumor cells harboring the E380Q mutation were associated with the lowest auto-activation rate of the estrogen receptor, and remained sensitive to an estrogen-mediated induction. In contrast, tumor cells harboring the Y537S mutation were related with a complete auto-activation of the estrogen receptor which could not be further activated by addition of estrogen. In the same line, fulvestrant provided a growth arrest in all mutant-derived xenografts (wild-type, E380Q, S463P, Y537C/N or D538G) except for the Y537S one (Toy et al. 2017). Back to clinics and in the same line, recent analysis of 195 patients included in the PALOMA-3 trial, which compared a fulvestrant + palbociclib combination versus fulvestrant + placebo after progression on endocrine therapy, showed that the Y537S mutation was the only *ESR1* mutation selected under fulvestrant, whatever the arm of treatment considered (O'Leary et al. 2018b). Taken together with those from the BOLERO-2 trial, these data suggest that Y537S mutation may be associated with a high-level of resistance to endocrine therapies. Thus, next generation of endocrine

therapies in development such as AZD9496 (Toy et al. 2017; Weir et al. 2016), GDC-0810 (Joseph et al. 2016), elacestrant (RAD1901) (Bihani et al. 2017) or bazedoxifene (Wardell et al. 2015) are tested against Y537S mutant-derived xenografts.

Since *ESR1* mutations are associated with acquired resistance on AI exposure, some studies have investigated the potential interest of monitoring *ESR1* mutation using repeated sampling under AI exposure, and one after exposure to a SERD of new generation (AZD9496). The first study reported a 75% detection rate and a lead-time detection of 3–6 months before clinical progression among the 20 patients with *ESR1* circulating mutation included. After progression on AI, the analysis of 33 patients with circulating *ESR1* mutation showed that if an increase of the mutation rate was always associated with progression of the disease, a decrease/disappearance of the mutation rate did not imply a control of the disease (Clatot et al. 2016). These results were confirmed prospectively in a cohort of 83 patients under first-line AI for advanced BC. Overall, 39/83 patients (56%) had a circulating *ESR1* mutation detected with a median time of 6.7 months before clinical progression (Fribbens et al. 2018). Another retrospective study reported that after progression on AI and during a fulvestrant ± pictilisib treatment, partial or complete responses were associated with a decrease of circulating mutations rate (*PI3KCA* and *ESR1*); while for stable or progressing diseases no clear evolution of circulating mutations rates was observed (Spoerke et al. 2016). In the same line, the study reporting circulating *ESR1* mutation status under AZD9496 exposure among 45 metastatic BC did not observe a clear relationship between these circulating mutation level and patient outcome (Paoletti et al. 2018). Thus, based on these data, *ESR1* circulating mutation monitoring should probably be restricted to patients with an ongoing AI exposure since these mutations are frequently subclonal and do not predict clinical outcome after end of selection pressure by AI (O'Leary et al. 2018a). In that context, the large randomized PADA-1 trial is currently ongoing (NCT03079011) and plans to include 1000 first-line metastatic BC patients. Initial treatment is a combination of AI + palbociclib. An every 2 months follow-up is performed, and in case of circulating *ESR1* mutation emergence (without clinical progression), patients are randomized between continuation of first-line treatment versus switch to a fulvestrant + palbociclib combination. Co-primary endpoints are safety of the first-line treatment until randomization and efficacy of the treatment in both arms after randomization.

Besides *ESR1* mutations, a pilot study reported the potential interest of a circulating *ESR1* methylation assessment (observed in <10% of the cases), since the *ESR1* methylation status could be associated with resistance to endocrine therapy (Mastoraki et al. 2018). Finally, *ESR1* fusion protein—which have been reported in 1% of advanced BC—can be detected in blood samples and are associated with endocrine treatment resistance (Hartmaier et al. 2018).

3.3.4 BRCA Mutated BC

BRCA1 or BRCA2 are involved in the repair of DNA double-strand breaks by homologous recombination. BRCA1 or BRCA2 germline mutations increase the risk of breast cancer and are the main factor of genetic susceptibility to BC (Skol et al. 2016). Since homologous recombination is altered in BRCA mutated cancer cells, inhibition of the single-strand break repair mechanism by using PARP inhibitors was shown to improve outcome in metastatic BRCA BC patients (Robson et al. 2017). Yet, resistance to PARP inhibitors finally occurs. A recent analysis of BRCA mutated metastatic BC patients by targeted ctDNA sequencing revealed that polyclonal putative reversion mutations may be acquired under PARP/platinum treatments and detected in ctDNA of two-fifths patients resistant to these treatments (Weigelt et al. 2017).

4 Limitations

Use of ctDNA as liquid biopsy is an old concept but this research field only recently took its rise: most references of this chapter have been published since 2013. Indeed, lack of sensitivity of global cfDNA quantification or LOH analyses initially limited the development of ctDNA analyses. In contrast, dPCR or NGS based assays improved the limit of detection of mutant allele fraction in the bloodstream (Diaz and Bardelli 2014) and allowed analysis of very low amount of plasma samples (Murtaza et al. 2013) or archival samples (Sefrioui et al. 2017). Nevertheless, several limitations undermine the use of ctDNA in breast cancer.

First, the high heterogeneity among BC (such as intrinsic subtypes or different driver genes involved) prevent from identifying a unique marker in ctDNA that could work for all. Recurrent mutations which are easily targetable by dPCR in ctDNA are observed in around 50% of luminal or HER2 enriched BC tumors, and *ESR1* mutations are restricted to 30% of the patients progressing on AI exposure. Even the use of targeted panel genes sequencing (Dawson et al. 2013; Garcia-Murillas et al. 2015; Riva et al. 2017) can only detect mutation if at least one of these genes are mutated, which is not necessarily the case. In the same line, whole exome sequencing does not guaranty the identification of a driver mutation. For example, in the landmark study by Dawson et al. only 30 of the 52 (56%) metastatic BC patients had genomic alterations suitable for monitoring. Thus, if ctDNA analysis was a better biomarker compared to CA 15-3 and CTC count among these 30 patients, fairly comparison between these biomarkers performances should be carried out on the entire population of 52 patients (Dawson et al. 2013).

Second, when a driver mutation is identified in primary tumor, correlation between primary tumors and matched plasma samples is observed with a specificity of 100%, but a sensitivity of around 80% (Bettegowda et al. 2014; Garcia-Murillas et al. 2015; Riva et al. 2017; Zhou et al. 2016) which is in line with results from other cancer types (Karachaliou et al. 2015; Kuo et al. 2014; Thierry et al. 2014). Thus, when combining failure of determining a genomic alteration suitable for

monitoring (20–25%) and lack of sensitivity (20%), the theoretical amount of patients that can be effectively monitored is to two-third. Even if technical advances may improve detection rates, ctDNA is to date associated with a significant risk of false-negative cases.

Third, due to the wide range of mutant allele fraction (MAF) found in ctDNA from one patient to another even with the same tumor stage (Bettegowda et al. 2014; Heidary et al. 2014), defining a cut-off value above which a MAF would have clinical consequences is challenging. Thus, most of the large studies reported the presence/absence of circulating mutations (Baselga et al. 2017; Chandarlapaty et al. 2016; Fribbens et al. 2016; Moynahan et al. 2017) or correlated intra-individual monitoring of MAF amount with clinical outcome (Clatot et al. 2016; Spoerke et al. 2016) but did not define MAF thresholds efficient over the entire population.

5 Conclusion

Breast cancer is a frequent and heterogeneous disease. Current management of BC is based on clinical and histological prognostic and predictive factors discovered decades ago and molecular classification of BC primary still based on gene-expression. Since few years, better understanding of BC genomics all over the natural history of cancer helped identify DNA alterations related to cancer behavior. High-sensitivity of dPCR and NGS allowed for non-invasive detection of such targets in the blood. Circulating *ESR1* detection for patients receiving AI is at the forefront of liquid biopsy research in BC. But several other convincing targets are already investigated in clinical trials, such as circulating *PIK3CA* or *HER2* mutations. Continuous improvement of technological resources will for sure overcome some of the limitations of the liquid biopsy approach. Furthermore, while collecting blood samples for circulating biomarkers analyses during clinical trials was the exception until few years ago, it is now the rule. Thus, a huge amount of blood samples will soon be available and will provide strong evidence for defining when and how circulating DNA analysis may improve BC management.

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Cell-Free DNA in the Management of Colorectal Cancer

Alexandre Harlé

1 Introduction

Colorectal cancer (CRC) is the fourth leading cause of cancer-related deaths worldwide in both men and women (Siegel et al. 2017). Surgery, radiotherapy and chemotherapy potentially associated to monoclonal antibodies (mAbs) are the most used therapeutics for the management of patients with CRC (Van Cutsem et al. 2016; Vogel et al. 2017).

Because of its low invasiveness and its ability to provide a more comprehensive molecular portrait of the tumour compared to tissue biopsy, the interest of liquid biopsy and the detection of cell-free DNA (cfDNA) in plasma has increased since the last few years.

2 Genomic Landscape of Cell-Free DNA in Patients with Colorectal Cancer and Concordance with Tissue Biopsy

Whereas genomic landscape of colorectal cancer is well known (Yaeger et al. 2018), only few data are available to know whether cfDNA sequencing of patients with CRC can detect genomic alterations at frequencies similar to those observed by tumour tissue sequencing. Most of studies focused on the detection of *KRAS*, *NRAS* and *BRAF* genes mutations with yielded concordance rates of 67 and 76% for the oldest studies (Ryan et al. 2003; Trevisiol et al. 2006) and more than 90% for studies published from 2014 (Bettegowda et al. 2014; Thierry et al. 2014).

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In a recently published article, next-generation sequencing data from cfDNA of 1397 patients with colorectal cancer have been compared to data from direct tissue sequencing. Frequencies of genomic alterations detected in cfDNA were comparable to those observed in three independent tissue-based colorectal cancer sequencing compendia (Strickler et al. 2018). Another recent exome sequencing study on both cfDNA and tissue, yielded a 77% concordance rate (Toledo et al. 2018). This data suggests that cfDNA analysis may be a good alternative to tissue sampling for the identification of the genomic properties of tumours in patients with CRC.

3 cfDNA as Diagnostic Marker

The golden standard strategy for diagnosis of CRC is faecal occult blood testing and colonoscopy followed by a histopathology examination of the biopsied suspect and potentially pre-cancerous lesions. Screening and colonoscopy are often rejected by the population and sensitivity as well as the specificity are not sufficient for early colorectal cancer detection. A blood-based test seems to be an easy and convenient surrogate tool for CRC mass-screening and early detection.

Most of blood-based tests described in the literature detect total cfDNA or ALU sequences detection (i.e. ALU115 and ALU247) but the discriminating power of these approaches seem limited for stage I and stage II CRC detection and adenomas (Normanno et al. 2018). In a meta-analysis published in 2018, 14 studies using total cfDNA or ALU sequences detection for CRC have been included (Wang et al. 2018). The analysis of data from 1258 patients with CRC and 803 healthy individuals showed a sensitivity of 0.735 (95% CI 0.713–0.757) and specificity of 0.918 (95% CI, 0.900–0.934); positive likelihood ratio was 8.295 (95% CI, 5.037–13.659) and negative likelihood ratio was 0.300 (95% CI, 0.231–0.391). Finally, diagnostic odds ratio was 30.783 (95% CI, 16.965–55.856). The authors concluded that diagnostic accuracy of cfDNA has unsatisfactory sensitivity but acceptable specificity for diagnosis of colorectal cancer.

Other studies have tracked the common somatic mutations described in CRC in cfDNA for early detection. Koperski et al. detected *KRAS* mutations in blood of 5/8 patients with CRC, 22/62 with adenoma but also 37/170 individuals without known neoplasia (Kopreski et al. 2000). Other studies have also reported that somatic alterations can be detected in cfDNA in 0.45–20% of healthy individuals (Normanno et al. 2018). The use of NGS assays like Safe-SeqS or TEC-Seq and extended genes panel should significantly improve the detection of somatic mutations in cfDNA of patients with early CRC. Safe-SeqS showed detection possible for stage I and stage II CRC in 47% and 55% respectively (Bettegowda et al. 2014). TEC-Seq technology showed to enable the detection of 50 and 89% of stage I and stage II CRC respectively (Phallen et al. 2017).

Detection in the cfDNA of aberrant patterns of methylation may represent a promising biomarker for early diagnosis of CRC. The detection of the methylated Septin 9 (^{m}SEPT9) in patients with CRC successfully identified 72% of Stages I–III cancers at a specificity of 93% in a preliminary study (deVos et al. 2009) but after approval of the test by the FDA, results were found to be not as good as expected. A second version of the assay showed that sensitivity and specificity were 74.8% (95% CI: 67.0–81.6%) and 87.4% (vs. non-CRC, 95% CI: 83.5–90.6%), respectively. The assay was positive in 66.7% of stage I, 82.6% of stage II, 84.1% of stage III, and 100% of stage IV CRCs (Jin et al. 2015). Combination of different methylated targets detection seems to improve sensitivity and specificity of the test, as well as the combination of the measurement of different parameters. The CancerSEEK assay is a multi-analytes blood test that includes cfDNA which can detect 8 different types of cancer in early-stage (Cohen et al. 2018). More than 65% early-stage CRC have been detected using this assay.

4 Prognosis for Patients with Non-metastatic and Metastatic CRC

In 2016, Tie et al., published the analysis of 1046 plasma samples from a prospective cohort of 230 patients with resected stage II colon cancer (Tie et al. 2016). Presence of postoperatively detectable cfDNA in plasma was associated with an inferior recurrence-free survival in patients not treated with adjuvant chemotherapy [hazard ratio (HR), 18; 95% confidence interval (CI), 7.9 to 40; $P < 0.001$] and also in patients treated with chemotherapy(HR, 11; 95% CI, 1.8 to 68; $P = 0.001$). The authors concluded that cfDNA detection after stage II colon cancer resection provides direct evidence of residual disease and identifies patients at very high risk of recurrence.

Interestingly, the meta-analysis by Basnet et al. showed that detection of cfDNA in plasma was associated to an inferior recurrence-free survival (HR [95% CI] = 2.78[2.08–3.72]) and overall survival (HR [95% CI] = 3.03[2.51–3.66]) in patients with CRC irrespective of disease stage, study size, tumour markers, detection methods and marker origin (Basnet et al. 2016). Several studies are indeed also published in patients with metastatic CRC and presence of cfDNA in plasma is always associated with shorter overall survival. El Messaoudi et al. showed on a 97 patients cohort with mCRC and a median 36 months follow-up, that presence of cfDNA in plasma was associated with a significantly shorter overall survival (18.07 months vs. 28.5 months, $p = 0.0087$) (El Messaoudi et al. 2016). This shorter OS in patients with mCRC with cfDNA detectable in plasma has also confirmed in almost all studies analysed in a meta-analysis by Fan et al. (2017).

5 Predictive Marker for Patients with mCRC

Anti-EGFR monoclonal antibodies (mAbs) associated to chemotherapy is now a standard for the first-line treatment of mCRC. Anti-EGFR mAbs can only be used in mCRC patients with *KRAS* and *NRAS* (*RAS*) wild-type tumour. Tissue is the golden standard for *RAS* testing in patients with mCRC, but liquid biopsy is now almost ready for prime time.

Several studies of RAS tissue/plasma status concordance using different assays like BEAMing, NGS or ddPCR are published. Most of them report a concordance over 90% (Bettegowda et al. 2014; Thierry et al. 2014) but are retrospective studies. In the prospective RASANC study, plasma samples from 425 chemotherapy-naive patients with mCRC were collected and analysed centrally by next-generation sequencing (NGS) and by methylation digital PCR (*WIF1* and *NPY* genes) (Bachet et al. 2018). Methylation assay has been used to determine whether cfDNA was present in the blood sample or not. Among the 425 enrolled patients, 412 patients had available paired plasma and tumour samples. The authors found a 0.71 κ coefficient [95% CI, 0.64–0.77] and 85.2% accuracy (95% CI, 81.4–88.5%). In the 329 patients with a positive methylation assay, thus potentially with detectable ctDNA, the authors described a 0.89 κ coefficient (95% CI, 0.84–0.94) and 94.8%c accuracy (95% CI, 91.9–97.0%). Interestingly, the authors found that the absence of liver metastases was the main clinical factor associated with inconclusive circulating tumour DNA results [odds ratio = 0.11 (95% CI, 0.06–0.21)]. In patients with liver metastases, accuracy was 93.5% with NGS alone and 97% with NGS plus the methylated biomarkers. The authors conclude that this prospective trial demonstrates excellent concordance between RAS status in plasma and tumour tissue from patients with colorectal cancer and liver metastases, thus validating liquid biopsy for routine *RAS* mutation testing in these patients. Data from other prospective studies like ColoBEAM (Harlé et al. 2019), using BEAMing assay, confirmed high overall tissue/blood concordance (83.2% with Se=77.3% and Sp=94.3%), and even higher tissue/blood *RAS/BRAF* concordance is 89.3% (Se=87.5%; Sp=92.0%) in chemotherapy-naive patients. The highest concordance was observed in chemotherapy-naive patients with liver metastasis (91.8%; Se=93.3%; Sp=89.5%).

These results of the ColoBEAM study confirm cfDNA extracted from plasma as a credible surrogate marker to tissue DNA biopsy for *KRAS*, *NRAS* and *BRAF* mutations assessment and that liquid biopsy has the potential to be incorporated as first-line theragnostic *RAS/BRAF* assessment especially in chemotherapy-naive patients with mCRC.

6 Monitoring Response to Therapy and Clonal Evolution of the Disease

Another promising application of cfDNA detection in plasma of patients with mCRC is response monitoring, and clonal evolution of the disease.

In 2012, Misale et al. described the emergence of *KRAS* mutation in blood 10 months prior recurrence detection using imagery of patients treated with anti-EGFR mAbs (Misale et al. 2012). The authors then suggested that liquid biopsy would be a good surrogate marker for resistance to treatment early detection.

In 115 patients with mCRC *RAS* mutated tumour and treated with chemotherapy-associated to anti-VEGF mAb, decrease of *RAS* variant allele frequency in patients has been described as associated to clinical benefit and early predictor of response, whereas no or low decrease were associated to shorter progression-free survival (Vidal et al. 2017). These results have been confirmed by the prospective PLACOL study with 82 patients with mCRC treated in first (82.9%) or second (17.1%) line chemotherapy (Garlan et al. 2017). The authors found that patients with a high (>10 ng/mL) versus low (≤ 0.1 ng/mL) cfDNA concentration at baseline (first chemotherapy) had a significantly shorter overall survival (6.8 vs. 33.4 months). The authors then classified the patients in 'good' and 'bad' responders by analysing the evolution of the cfDNA concentration between baseline and after first or second chemotherapy. Objective response rate has been found significantly better in 'good' responders; median progression-free survival was significantly longer (8.5 vs. 2.4 months: HR, 0.19; 95% CI, 0.09–0.40; $P < 0.0001$) as well as overall-survival (27.1 vs. 11.2 months: HR, 0.25; 95% CI, 0.11–0.57; $P < 0.001$).

Methylation can also be used for mCRC comprehensive monitoring of treatment response. In an article published in 2017, Barault et al., studied the methylation of a 5-gene panel (*EYA4*, *GRIA4*, *ITGA4*, *MAP3K14-AS1* and *MSC*) using a digital PCR assay in cfDNA from patients with mCRC ($n = 182$) (Barault et al. 2017). Plasma longitudinal assessment was performed in a patient subset treated with chemotherapy or targeted therapy. The authors found that methylation in at least one marker was detected in 156 cfDNA samples (85.7%). Plasma marker prevalence was 71.4% for *EYA4*, 68.5% for *GRIA4*, 69.7% for *ITGA4*, 69.1% for *MAP3K14-AS1* and 65.1% for *MSC*. Methylation markers were not affected by treatment type and correlated with objective tumour response and progression-free survival.

One of the remarkable application of monitoring cfDNA in plasma of patients with mCRC is the possibility of therapy rechallenge. The study published by Siravegna et al. (2015) demonstrated for the first time that the CRC genome adapts dynamically to intermittent drug schedules and provide a molecular explanation for the efficacy of rechallenge therapies based on EGFR blockade. In this study, five patients treated with anti-EGFR mAbs were found with an emerging *KRAS* mutation when analysing cfDNA extracted from plasma and one patient developed a *MET* amplification. This data suggests that some new tumour clones bearing a *KRAS* mutation were emerging in these patients at the time of disease progression. These mutated clones declined when treatment with anti-EGFR mAbs was suspended and substituted with another chemotherapy treatment line, which allowed to rechallenge anti-EGFR mAbs in the further line of treatment. This important data shows that lines of treatment could be re-used in the same patient, drastically changing the actual practice for the management of patients with CRC.

7 Future Perspectives

Use of cfDNA in the management of CRC is still improving and new promising perspectives are about to be validated. The development of NGS assays like Safe-SeqS, TEC-Seq or CAPP-Seq will probably help to reduce background noise in the measurement of cfDNA in plasma, thus allow a most precise measurement of residual disease in patients treated for CRC or emergence of subclones. A study published by Van Emburgh et al. (2016) demonstrated that clonal evolution during the acquisition of resistance impacts the clinical response to anti-EGFR mAbs in patients with CRC. The authors hypothesised that this may be influenced by the subclonal mutational landscape and environmental pressure on the tumour.

Another application of wide-range NGS is the molecular classification of CRC using only liquid biopsy. Four classifications are admitted in the gene expression–based consensus molecular subtypes (CMS) and provide a new paradigm for stratified treatment. CMS1 which are hypermutated, microsatellite unstable and have a strong immune activation, CMS2 which are epithelial with a marked WNT and MYC signalling activation; CMS3 which are epithelial and have evident metabolic dysregulation, and CMS4 which have a prominent transforming growth factor-β activation, stromal invasion and angiogenesis (Guinney et al. 2015). This classification is only based on tissue analysis, but recent data suggests that all these parameters could be easily assessed using NGS. In a recent presentation at ASCO 2018, NGS has been described as suitable for the determination of MSI status using cfDNA extracted from plasma (Barzi et al. 2018). The analysis of cfDNA has also been proven to be a relevant tool for the screening of actionable mutations in various cancers including CRC (Zill et al. 2018). The detection of actionable alterations using only liquid biopsy is particularly important for the management of patients with multiple recurrences in a tumour board context for the selection of off-label targeted therapies that may be effective on the present clones.

8 Conclusions

cfDNA is a promising tool for the management of patients with CRC, for early detection, diagnosis, prognosis, as a predictive marker and for response to treatment monitoring or minimal residual disease assessment. The low-invasiveness and ease of iterative sampling make cfDNA the perfect surrogate to tissue biopsy. Moreover, it is admitted today that cfDNA is the mirror of tumour heterogeneity, which makes liquid biopsy a more comprehensive tool than a simple biopsy of only one region of the tumour. The final barrier for the use of cfDNA in clinical routine for the management of patients with CRC is the lack of prospective randomised clinical trials. Several cfDNA-based clinical trials are recruiting thus this major issue should be solved in the next few years.

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Dynamic Treatment Stratification Using ctDNA

Joana Vidal, Alvaro Taus and Clara Montagut

1 Introduction

Despite great improvements in prevention, detection, and treatment, cancer still is one of the leading causes of death worldwide (Siegel et al. 2017). One of the seminal advances in the treatment of cancer has been the identification of clinically actionable genetic aberrations. Targeting of oncogenic drivers such as *CKIT* in GIST; *HER2* in breast cancer; *EGFR*, *ALK* or *ROS1* in non-small cell lung cancer (NSCLC); or *BRAF* in melanoma has resulted in relevant advances for the treatment of several malignancies. In other cases, such as in metastatic colorectal cancer (mCRC), is the absence of mutations in the *RAS* gene that predicts response to anti-EGFR treatments (Schwaederle et al. 2015).

The genomic characterization of tumor tissue has been established as routine practice in oncology. However, obtaining tumor tissue through biopsy has several limitations, mainly related with the invasiveness of the procedure and a biased representation of tumor heterogeneity. Detection of mutations in circulating tumor (ct) DNA has been postulated in recent years as an alternative to classical tumor biopsy both at the time of diagnosis and for treatment monitoring (Diaz and Bardelli 2014).

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In the last decade, the comprehensive characterization of cancer genomic landscape together with the implementation of highly sensitive sequencing technologies have permitted the implementation of ctDNA-based liquid biopsy as a tool that is changing the diagnosis, treatment, and follow-up of cancer.

2 ctDNA to Monitor Response to Systemic Treatment

Tumor burden and response to treatment are evaluated in clinical practice by the Response Evaluation Criteria In Solid Tumors (RECIST) 1.1, which measures the diameter variations of tumor lesions using imaging tests. This is often accompanied by blood protein biomarkers such as CEA, CA15.3, AFP, PSA or CA125. The lack of sensitivity and specificity of protein biomarkers (Bettegowda et al. 2014) together with the limitations of the RECIST criteria to evaluate the response to new biological therapies (Seymour et al. 2017) demonstrates the need of an effective and reproducible method to assess therapeutic response in cancer patients. Moreover, it would be preferable to continuously monitor therapeutic response rather than wait for imaging assessments performed every 8–12 weeks. Several studies have demonstrated how ctDNA can be a surrogate marker of tumor burden during systemic treatment in patients with advanced disease. The high turnover of ctDNA, which has a half-life of a few hours, makes it a useful tool to provide real-time information on the evolution of a singular patient.

In 2013, the first large ctDNA monitoring study was published in patients with metastatic breast cancer (Dawson et al. 2013). The authors used targeted deep sequencing techniques to screen for point mutations in *TP53* and *PIK3CA* in tissue biopsy from 52 patients. In 30 cases genomic alterations were identified and subsequently, a personalized panel was designed to quantify the mutations detected in the plasma of each patient. Variations in ctDNA were compared to levels of the biomarker CA15.3. ctDNA was detected in 97% of patients while CA15.3 was only elevated 78% of women. A higher dynamic variation was observed in the ctDNA compared to the CA15.3 levels, which correlated with changes in tumor volume throughout follow-up. Increases in ctDNA levels reflected progressive disease in 17 of 19 women and, in 53% of cases, levels of ctDNA increased on average 5 months before the establishment of progressive disease by means of imaging. The utility of ctDNA was also explored in patients with advanced breast cancer who develop resistance to Aromatase Inhibitors (AI) by acquiring ESR1 mutations. Schiavon and colleagues developed an ultrahigh-sensitivity multiplex digital polymerase chain reaction assays for ESR1 mutations in ctDNA (Schiavon et al. 2015). Patients with ESR1 mutations had a substantially shorter progression-free survival (PFS) on subsequent AI-based therapy (HR 3.1; 95% CI, 1.9 to 23.1; $p = 0.0041$). Taking this data in account, O'Leary and colleagues analyzed plasma samples from the 455 patients enrolled in the randomized phase III PALOMA-3 study of CDK4/6 inhibitor palbociclib and fulvestrant for women with advanced breast cancer (O'Leary et al. 2018). ctDNA showed how a relative change in *PIK3CA* ctDNA level after

15 days of treatment strongly predicted PFS on palbociclib and fulvestrant (HR 3.94, $p = 0.0013$). *ESR1* mutations selected by prior hormone therapy were shown to be frequently subclonal, with *ESR1* ctDNA dynamics offering limited prediction of clinical outcome (Figs. 1 and 2).

Similar results were obtained in 20 patients with advanced $BRAF^{V600E}$ mutated melanoma treated with BRAF inhibitors (Sanmamed et al. 2015). Basal concentrations of circulating $BRAF^{V600E}$ were correlated with tumor burden, and a decline on ctDNA fraction during the first month of treatment was linked to subsequent response. At the time of progression, an increase in circulating $BRAF^{V600E}$ concentration was detected compared to ctDNA levels from responding patients. In addition, it has been described how lower basal levels of circulating $BRAF^{V600E}$ were significantly associated with a greater overall survival (OS) and PFS. In another study published by Lipson et al. (2014), twelve patients undergoing treatment with immunotherapy blockade were monitored using ctDNA. Authors analyzed specific hotspot mutations prevalent in melanoma such as *BRAF*, *CKIT, NRAS* or *TERT*. ctDNA levels were correlated with clinical and radiological outcome and, in one patient, preceded eventual tumor progression.

One of the first studies in colorectal cancer (CRC) was the one published by Tie et al. (2015). A panel of 15 genes in 53 patients with mCRC who received first-line systemic treatment was evaluated. At least one mutation was detected in tissue from 92% of patients, which was later monitored in plasma by dPCR techniques. A significant reduction in ctDNA levels was observed prior to the second cycle of treatment which correlated with the radiological response on CT performed at

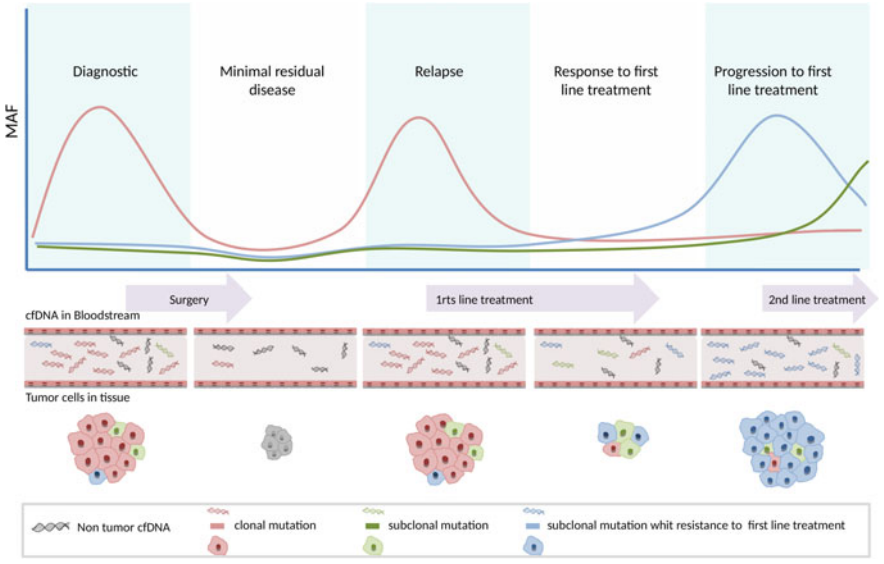

Fig. 1 Clinical applications of ctDNA analysis

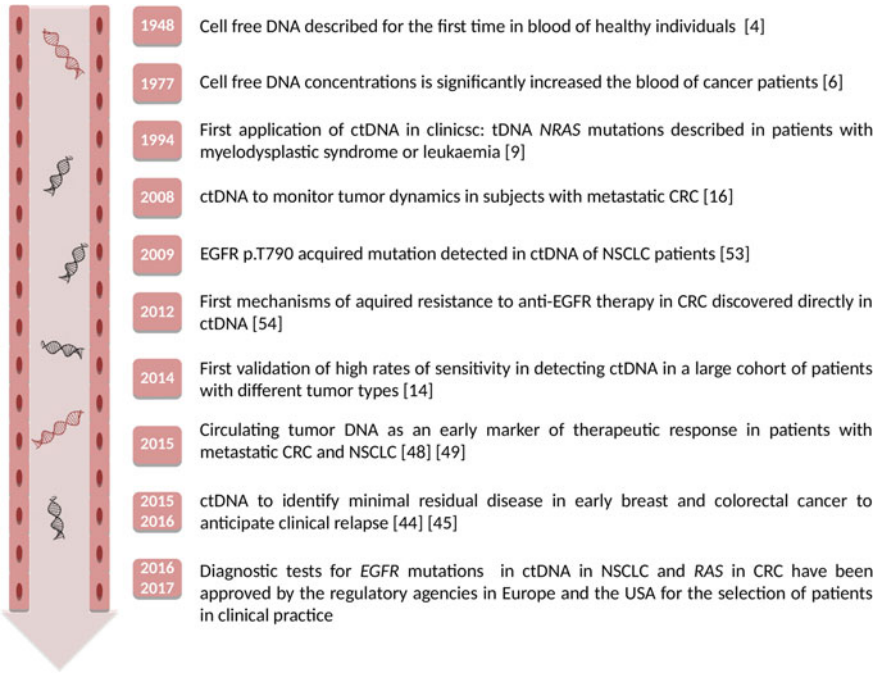

Fig. 2 Timeline of principal advances in ctDNA

8–10 weeks. Higher reductions in ctDNA concentration (>10-fold change) were associated with a trend towards increased PFS, although not statistically significant.

In an effort to explore the capacity of ctDNA to reflect tumor load in mCRC Vidal and colleagues examined the utility of tracking *RAS* mutations by OncoBEAM *RAS* CRC ctDNA testing to monitor the response of patients to treatment (Vidal et al. 2017). Serial blood draws were extracted from 21 patients with baseline *RAS* mutations undergoing systemic therapy (chemotherapy ± antiangiogenic). Analysis of *RAS* ctDNA at the time of a first CT scan (8–12 weeks of treatment) revealed a dramatic decrease in plasma *RAS* mutant allele fraction (MAF) in responding patients. MAF percentage of change was significantly lower in patients that progressed at first evaluation of response compared with patients with clinical benefit (132% increase vs. 99% reduction, respectively, p 0.027). In addition, we analyzed the prognosis impact of basal MAF levels in a cohort of 22 patients with at least 3 years of follow-up. Patients with MAF levels $\geq 1\%$ had significant worse prognosis than those with basal levels <1% (median PFS 17.6 month for patients with MAF <1% vs. 7.2 months in those patients with MAF $\geq 1\%$ $p = 0.44$ and OS 47.6 month vs. 19.7 month, respectively $p = 0.038$). These data, supported by other similar studies (Morelli et al. 2015), suggest that ctDNA levels could also provide valuable information to predict the disease evolution in *RAS* mutant patients prior to treatment onset.

The utility of ctDNA analysis in monitoring thera-peutic response has also been reported in patients with NSCLC harboring EGFR mutations. In 2015, Marchetti et al. (2015) analyzed EGFR levels variations in serial plasma samples from 69 patients under treatment with erlotinib. The decline of circulating EGFR concentration was correlated with tumor response. This decrease was already evident 14 days after initiating treatment in fast responders, whereas in 2 patients with no clinical response an early increase of the $EGFR^{T790M}$ resistance mutation was observed. Recently Taus and colleagues confirmed these data in 221 plasma samples from 33 patients (Taus et al. 2018). *EGFR* mutations in plasma were detected in 83% of all patients and 100% of those with extra-thoracic metastases. The dynamics of the EGFR mutational load predicted response in 93% and progression in 89% of cases well in advance of radiologic evaluation. PFS for patients in whom ctDNA was not detected in plasma during treatment was significantly longer than for those in whom ctDNA remained detectable (295 vs. 55 days; HR 17.1; $p < 0.001$).

Subsequently, similar studies with second- and third-generation EGFR-TKIs such as afatinib and osimertinib have confirmed that rapid decrease or disappearance of EGFR mutation levels in ctDNA predicts response to treatment and higher PFS (Iwama et al. 2017; Oxnard et al. 2016).

The main conclusion of all these studies is the evidence that variation of ctDNA levels correlates and often anticipates the therapeutic response assessed by morphological criteria.

3 ctDNA to Monitor Response to Immunotherapy

Recent clinical results support the use of Immunotherapy with antibody checkpoint inhibitors such as anti-PD-1 (e.g., nivolumab, pembrolizumab) and anti-PD-L1 (e.g., atezolizumab), or anti-CTLA4 (ipilimumab) as part of the antitumor treatment in several cancer types. Delayed tumor shrinkage is frequently observed and can sometimes be preceded by transient increase of the diameter of tumor lesions due to immune cell infiltration (pseudo-progression). There is, therefore, an urgent need to identify biomarkers that accurately predict for treatment response and avoid discontinuation of a potentially effective therapy. Currently, the tumor-associated expression of PD-L1 and the distribution and density of tumor-infiltrating lymphocytes (TILs) are commonly examined in immune therapy based trials. The clinical utility of these markers is limited due to inter- and intra-patient heterogeneity (Madore et al. 2015), and they may require repeat, invasive biopsies. Moreover, in melanoma patients, response is independent from PD-L1 expression in solid tissue.

Lee and cols. recently assessed the role of ctDNA in late-stage melanomas treated with anti-PD1 based immunotherapy (Lee et al. 2017). Baseline and longitudinal assessment of ctDNA levels, including BRAF and NRAS mutations were analyzed. Authors described three distinct patient profiles. Profile Group A ($n = 36$)

consisted of patients with undetectable ctDNA levels at baseline and during therapy. Group B (n = 22) had detectable baseline ctDNA but became undetectable early during therapy. Group C (n = 18) had detectable ctDNA at baseline and during therapy. Undetectable ctDNA levels at baseline or within 8 weeks of therapy were predictive of response and prognosis being superior to other clinical classic biomarkers including LDH, disease burden and ECOG performance status.

Similar results were presented in a small study with 15 patients with non-small cell lung cancer, uveal melanoma or microsatellite-instable colorectal cancer treated by nivolumab or pembrolizumab (Cabel et al. 2017). At week 8, a significant correlation (r = 0.86; p = 0.002) was observed between synchronous changes in ctDNA levels and tumor size. Patients in whom ctDNA levels became undetectable at week 8 presented a marked and lasting response to therapy.

Dynamic ctDNA levels were also studied in three clinical trials of advanced melanoma treated with activated autologous TIL's (Xi et al. 2016). $BRAF^{V600E}$ ctDNA levels were analyzed by a sensitive allele-specific PCR assay in 388 serum samples from 48 patients and authors correlated differences in the dynamic patterns of their ctDNA measurements with response outcomes. All patients except one who achieved a CR developed a peak of mutant V600E ctDNA early during TIL treatment (in most patients, between days 5 and 9), and all showed early initial clearing of mutant DNA in serum. All but one of the CR patients continued to show no evidence of V600E ctDNA during follow-up studies, up to 8 years.

In NSCLC efforts are also being made to correlate the clinical response to immune checkpoint inhibitors and ctDNA. Goldberg et al. recently published the results on longitudinal changes in ctDNA levels compared to changes in radiographic tumor size and clinical outcomes from 28 patients with metastatic NSCLC under anti-PD1 or anti-PD-L1 treatment (Goldberg et al. 2018). ctDNA was quantified by determining the allele fraction of cancer-associated somatic mutations in plasma using a multi-geneNGS assay. Patients with >50% ctDNA MAF decrease from baseline was strongly associated radiographic response (Cohen's kappa, 0.753) and superior PFS [hazard ratio (HR), 0.29; 95% CI, 0.09–0.89; P = 0.03], and OS (HR, 0.17; 95% CI, 0.05–0.62; P = 0.007).

Similar results were obtained from a little study with 14 patients with NSCLC treated with nivolumab (Iijima et al. 2017). From 7 out of 14 patients, ctDNA mutations were detected at baseline (corresponding to those patients with higher tumor burden). Basal and serial ctDNA analysis revealed that a decrease in MAF of ctDNA showed high-level correlation with a durable response.

All these data reflects a congruent relationship between rapid and profound ctDNA changes and response to immunotherapy. These results are in clear contrast to the typically more modest radiographic reductions in tumor bulk seen within the same time-frame. A likely explanation is that ctDNA levels reflect the rate of active tumor cell death, rather than total tumor mass. Confirming those promising results with prospective and larger studies will be needed to implement ctDNA in routine clinical practice in patients treated with immunotherapy.

4 ctDNA for the Detection of Clonal Dynamics and Tumor Heterogeneity

Cancer is an evolutionary disease governed by clonal selection. Anti-cancer drugs exert a selective pressure that modifies tumor evolution by selecting resistant pre-existing clones that continue to grow despite treatment pressure, allowing for tumor progression. Defining the appearance of these resistances is essential when choosing the most appropriate treatment for each patient, as well as to avoid continuing ineffective therapies with unnecessary side effects. Recent studies have shown how ctDNA can be used to monitor the emergence of multiple resistance clones in the course of treatment.

One of the first described mechanisms of acquired resistance to targeted therapy was the emergence of $EGFR^{T790M}$ mutation in up to 50% of EGFR-mutated NSCLC patients treated with EGFR-TKI (Pao et al. 2005). These results, initially described in tissue samples from patient's re-biopsies, were subsequently confirmed in ctDNA by Taniguchi et al. (2011), providing the first example that resistance to targeted therapies in solid tumors can be detected non-invasively in patients' blood. Recently, the detection in plasma of $EGFR^{T790M}$ in EGFR-TKI-refractory patients has been shown to predict response to osimertinib equivalently than tissue biopsy determination (Oxnard et al. 2016). Although the efficacy data, the positive agreement between plasma and tissue was 76.7% meaning that about 1 out of 4 patients with $EGFR^{T790M}$ positive in tissue no plasma mutation was detected. The authors suggest the complementary role of both techniques, where plasma genotyping would be the first step to be performed and tumor biopsy would be complementary in case of obtaining a negative result in ctDNA, thus avoiding unnecessary invasive biopsies to patients. Based on these results, two tests are approved to detect emergence of $EGFR^{T790M}$ in NSCLC (Therascreen EGFR RGQ PCR Kit and Cobas EGFR Mutation test) and international clinical guidelines (REF NCCN NSCLC) nowadays strongly recommend ctDNA analysis at progression to anti-EGFR-TKI becoming a new routine test for those selected patients.

Similarly, patients with mCRC *RAS* wild-type who initially respond to anti-EGFR monoclonal antibodies (cetuximab or panitumumab) eventually develop resistance, which is mainly due to the emergence of mutations in MAPK pathway (*KRAS, NRAS, BRAF, MEK* genes) (Misale et al. 2012; Diaz et al. 2012), mutations in the EGFR extracellular domain (EGFR ECD) (Montagut et al. 2012; Arena et al. 2015), or activation of alternative pathway (HER2 or cMET) (Yonesaka et al. 2011; Bardelli et al. 2013). Several studies reported the detection of these mutations simultaneously in tissue and plasma of patients at the time of treatment progression and, interestingly, one study shows how the emergence of resistant KRAS-mutated clones could also be detected up to 10 months before radiographic evidence of progression (Misale et al. 2012; Diaz et al. 2012). Indeed, Diaz et al. (2013) described, by using a mathematical model, how baseline *RAS* mutant subclones pre-exist in a small fraction of the tumor, and increase under therapeutic pressure. Interestingly, while tissue biopsies detected one demonic alteration of acquired

resistance mechanism, ctDNA was capable to catch the complexity of tumor heterogeneity and detect multiple mutations in *RAS* and EGFR ECD coexisting in the same patient after anti-EGFR therapy (Siravegna et al. 2015; Van Emburgh et al. 2016). Moreover, in *RAS* wt CRC patients treated with FOLFIRI-cetuximab, the emergence of high concentrations of *RAS* mutations in plasma anticipates tumor progression as well as a rapid clinical deterioration, while continued *RAS* wt circulating status correlates with a prolonged response (Toledo et al. 2015).

A recent phase II trial in mCRC patients analyzed the efficacy of SYM004 (a mixture of 2 nonoverlapping monoclonal antibodies targeting EGFR) at anti-EGFR progression (Montagut et al. 2018). The authors performed a baseline ctDNA profile of 70 genes from 193 patients included by using the digital NGS Guardant360 (Guardant Health) and monitored ctDNA EGFR ECD mutations. The ctDNA analysis described a subgroup of patients *RAS* wild-type, BRAF wild-type and EGFR ECD wild-type (called triple-negative mCRC) who showed clinical improvement in median OS with SYM004. On the contrary, although a decrease in EGFR ECD MAF was observed under SYM004 therapy, no clinical benefit was observed in patients with ctDNA EGFR ECD mutations, in part due to heterogeneity and subclonal nature of EGFR ECD mutations. Further studies will prove whether ctDNA is useful to select further lines of treatment following failure to anti-EGFR therapy in mCRC patients.

In mCRC patients resistant to anti-EGFR therapy and emergence of a KRAS mutation, withdrawal of anti-EGFR therapy correlated with a decline of *KRAS*-mutant allelic fraction in ctDNA (Siravegna et al. 2015). In this setting, ctDNA could be used to measure *RAS* mutations in plasma and guide precise administration of anti-EGFR therapy holidays and rechallenge. The CRICKET clinical trial assessed the benefit of re-introducing cetuximab after treatment interruption in patients that responded to cetuximab in the first-line setting (Cremolini et al. 2018). Interestingly, retrospective ctDNA analysis showed that *RAS* mutant patients, as defined by ctDNA before rechallenge, did not benefit from cetuximab re-introduction. Other ongoing clinical trials such as CHRONOS and FIRE-4 are evaluating the use of ctDNA to assess *RAS* washout and guide rechallenge with cetuximab/panitumumab.

These findings illustrate how ctDNA allows for a complete picture of the tumoral heterogeneity and clonal evolution that provides the clinician comprehensive information of the complex molecular landscape. This is crucial to guide clinical decisions in the personalized era of cancer treatment.

5 Conclusions

In recent years, advances in the understanding of tumor biology and the emergence of novel targeted therapies have contributed in extending the survival of patients with cancer. Moreover, deeper knowledge on clonal dynamics and tumor heterogeneity has made imperative a real-time characterization of the genomic landscape of cancer.

Serial biopsies of tumor tissue have been used so far, involving technical and logistical limitations together with potential comorbidities and biased representation of heterogeneity. The improvement of sequencing technologies has allowed for the detection of mutations in ctDNA from a peripheral blood extraction, and since then, several clinical applications of liquid biopsy have been exploited. Liquid biopsies can be particularly advantageous for molecular diagnosis in the metastatic setting as a safe minimally-invasive alternative to tissue. Besides, ctDNA testing has been shown to accurately represent intratumor and intertumor molecular heterogeneity.

ctDNA analysis permits a global, dynamic and real-time description of the tumor's molecular landscape, with a clear potential in the early detection of acquired resistance mutations that arise during treatment preceding evidence of clinical or radiological progression. These findings can guide the clinicians to avoid continuing the administration of non-effective treatments and develop new therapeutic approaches to improve the outcome of patients.

Although the implementation of ctDNA study is a reality in clinical practice—especially in mCRC, NSCLC and melanoma—it is essential to expand the evidence to other tumor types, as well as in other scenarios where very low levels of cDNA are detected (i.e., early stages and minimal residual disease). Currently, several prospective clinical trials in patients with solid tumors incorporate longitudinal ctDNA genotyping to monitor clonal dynamics and to guide treatment decisions. These studies are crucial to establish the clinical applicability of ctDNA in metastatic and localized solid malignancies, while future studies of ctDNA as a tool for screening and detection of pre-malignant disease are warranted.

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Circulating Tumor RNA, Exosomes

Circulating miRNAs as Biomarker in Cancer

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Abbreviations

EDTA	Ethylenediaminetetraacetic acid
HDL	High-density lipoproteins
LDCT	Low-dose computed tomography
MGCT	Malignant germ cell tumors
miRNA	MicroRNA
MSC	miRNA signature classifier
Nt	Nucleotides
qPCR	Quantitative polymerase chain reaction
snoRNA	Small nucleolar RNA
UTR	Untranslated region

The discovery of microRNAs (miRNAs) and their regulatory function in gene expression generated a shift in the understanding of the mechanisms involved in the transition of healthy cells into pathological disease states. Furthermore, the identification of circulating miRNAs in biofluids opened up new opportunities for replacing invasive tests with non-invasive "liquid biopsies." In this chapter, we present the recent expertise within the field of using circulating miRNAs as cancer biomarkers. We will, in particular, discuss the challenges within the field and list

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the variables in the pre-analytical, analytical, and post-analytical procedures, which may influence the findings when identifying new cancer biomarkers based on circulating miRNA.

1 miRNA Biogenesis and Its Deregulation in Cancer

miRNAs are part of a class of small non-coding RNA molecules (22–25 nucleotides long) regulating gene expression post-transcriptionally. They bind to complementary sequences usually in the 3′ untranslated region (UTR) of the mRNA for mammalian miRNAs (Stark et al. 2003). Cleavage of the mRNA is promoted if the base pairing is perfect or near perfect. However, mammalian miRNAs often have imperfect base pairing, which only leads to inhibition of the mRNA translation. Imperfect binding may, however, also lead to faster mRNA degradation, due to accelerated deadenylation (Williams 2008). The binding of a miRNA to its target mRNA thereby results in either mRNA cleavage or inhibited translation, causing reduced protein expression of the target gene. More than 2500 distinct miRNAs have so far been identified in humans (Kozomara and Griffiths-Jones 2011). Each microRNA can directly or indirectly regulate several hundred target genes and thereby regulate key cellular processes such as proliferation, differentiation, DNA repair, and apoptosis (Brennecke et al. 2005; Lewis et al. 2005; Afonso-Grunz and Muller 2015). Thus, changes in the miRNA synthesis can have a large impact on neoplastic initiation and progression, as it may affect the expression of both tumor suppressors and oncogenes (Lin and Gregory 2015). Additionally, miRNAs themselves also function as classical oncogenes/oncomiRs and tumor suppressors, as alterations of specific miRNAs have been shown to promote carcinogenesis (Lin and Gregory 2015; He et al. 2005; Chang et al. 2007; Wang 2010). However, several miRNAs exhibit both oncogene/tumor suppressor activity depending on the type of cancer or the cellular context (Svoronos et al. 2016). For instance, miR-125b acts as an oncomiR for most hematologic malignancies, whereas it acts for numerous solid tumors as a tumor suppressor (Shaham et al. 2012; Sun et al. 2013). This discrepancy, for specific miRNAs, can be explained by the potential capacity of a single miRNA to regulate several hundred mRNAs. The action of miRNAs depends thus on the cell-type-specific mRNA expression profile. Depending on which targets are present or differentially expressed through other molecular mechanisms such as chromosomal gains or deletions, the balance of the gene regulatory network will shift and target transcripts that may have tumor promoting or repressive functions. Deregulation of miRNA expression has been thoroughly described for the majority of cancer types for both tumor initiation and progression (Calin and Croce 2006; Negrini et al. 2009; Lee and Dutta 2009; Garzon et al. 2006), and for metastasis formation (Zhang et al. 2010) [for a comprehensive review of miRNAs and their functions in cancer see Berindan-Neagoe et al. (2014)]. This fundamental role of miRNAs in tumorigenesis emphasizes their potential use for diagnostic, prognostic, and therapeutic means.

2 miRNAs as Diagnostic Markers in Cancer

The use of miRNAs in clinical diagnostics of cancer has been accelerated by the description of specific miRNA profiles discriminating specific cancer types/subtypes (Lu et al. 2005; Volinia et al. 2006; Rosenfeld et al. 2008). Several tests using miRNA expression profiles for classification of various cancers have been developed for clinical use. The molecular diagnostics company Rosetta Genomics™ (USA) has developed a miRNA panel for the identification of the unknown primary origin of metastatic cancers (Meiri et al. 2012; Pentheroudakis et al. 2013). This assay uses a panel of 64 miRNAs to discriminate 49 different cancer types. Rosetta Genomics™ has also developed miRNA profiles/tests for the differentiation of histological subtypes of three different cancer types (thyroid, lung and kidney) (Benjamin et al. 2016; Lithwick-Yanai et al. 2017; Gilad et al. 2012; Spector et al. 2013). These tests depict the great potential of using miRNAs as biomarkers in cancer. However, the analyses of these tests are all based upon tumor biopsies, requiring invasive testing and are not amenable with the needs of a clinical biomarker requiring repeated sampling. To overcome this problem, an intense research focus within the last decade has been on developing non-invasive tests, such as "liquid biopsies" for predictive, diagnostic, prognostic, and therapeutic tests. This development is similar compared to the research effort on the analysis of mutations present at low proportions in biological material that has recently attracted great interest to detect clinically relevant subpopulations or to follow the response and potential emergence of mutations conferring treatment resistance (Wan et al. 2017; Heitzer et al. 2015). However, the mutations to be followed need to be adapted to each individual patient and new subclones with genetic mutations may arise and escape detection. MiRNAs with their small size and conventional qPCR-based detection methods might therefore present an alternative tool for patient management.

3 Circulating miRNAs in Biofluids

The anticipation of developing non-invasive tests using biofluids was greatly motivated by the discovery of tumor-specific circulating miRNAs in blood a decade ago (Lawrie et al. 2008; Mitchell et al. 2008; Chen et al. 2008). Since then, detection of circulating miRNAs has been confirmed for numerous types of human biofluids, including serum, plasma, urine, saliva, tears, and cerebrospinal fluid (Weber et al. 2010). The transportation of miRNAs in biofluids has been described in at least two ways. They can be packaged and transported in exosomes and other cell-derived extracellular vesicles containing miRNAs, mRNAs, and proteins (Valadi et al. 2007; Smalheiser 2007; El-Hefnawy et al. 2004; Wang et al. 2010). These have been identified in several biofluids, such as blood, saliva, and urine (Hunter et al. 2008; Michael et al. 2010; Keller et al. 2011). Another mechanism of miRNAs entering into and being transported in biofluids is by binding and secretion

of the miRNA to protein complexes. One study has demonstrated that potentially 90% of the circulating serum and plasma miRNAs are not encapsulated by exosomes, but bound to protein complexes such as Argonaute proteins (Ago2) (Arroyo et al. 2011). Other protein complexes shown to bind and transport miRNAs in biofluids are high-density lipoproteins (HDL) and the RNA-binding protein nucleophosmin 1 (NPM1) (Wang et al. 2010; Vickers et al. 2011) [a thorough specification of the transport and possible biological functions of circulating miRNAs is described in Cortez et al. (2011)]. The discovery of circulating miRNAs enabled new avenues for the use of miRNAs as both diagnostic and prognostic biomarkers.

4 Advantages of Using Circulating miRNAs as Biomarkers in Cancer

The enthusiasm of identifying circulating miRNAs as biomarkers was further reinforced by the discovery of their exceedingly high stability in circulation and their resistance to RNase degradation (Mitchell et al. 2008; Chen et al. 2008; Mall et al. 2013; Ishikawa et al. 2017). Serum miRNA levels remain stable after exposure to severe conditions such as boiling, extended storage, ten freeze–thaw cycles, and extreme pH levels (Chen et al. 2008; Ishikawa et al. 2017). Additionally, circulating miRNAs have a very robust preservation, as 10-year-old human serum samples and dried serum blots stored at room temperature still contained a vast number of miRNAs (Zhu et al. 2009; Patnaik et al. 2010). The high stability is one of the main characteristics of what makes circulating miRNAs superior as biomarkers, but they essentially fulfill all criterions of a biomarker. The official definition of a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group 2001). Therefore, an ideal cancer biomarker must have a distinct profile between the group of interest and a control group (such as cancer patients vs. healthy individuals; patients with metastatic disease vs. patients with a localized tumor; and patients with a poor response to a given chemotherapy vs. patients with a good response to the same chemotherapy). Furthermore, an ideal biomarker should be easy to obtain through non-invasive methods, analyzed by simple and inexpensive methods and be stable in clinical samples over a long period of time. All these prerequisites are fulfilled by circulating miRNAs, as their expression can be determined in a biofluid sample (blood, urine, saliva, etc.) by simple methods already established in the clinic such as quantitative PCR (qPCR) or next-generation sequencing.

The pronounced enthusiasm for the potential use of circulating miRNAs as cancer biomarkers is also reflected by the magnitude of annual publications in the PubMed database. This field of research has had an almost exponential increase within the last decade, both in regard to papers investigating the general implication of miRNA deregulation within the field of tumorigenesis, but also for the

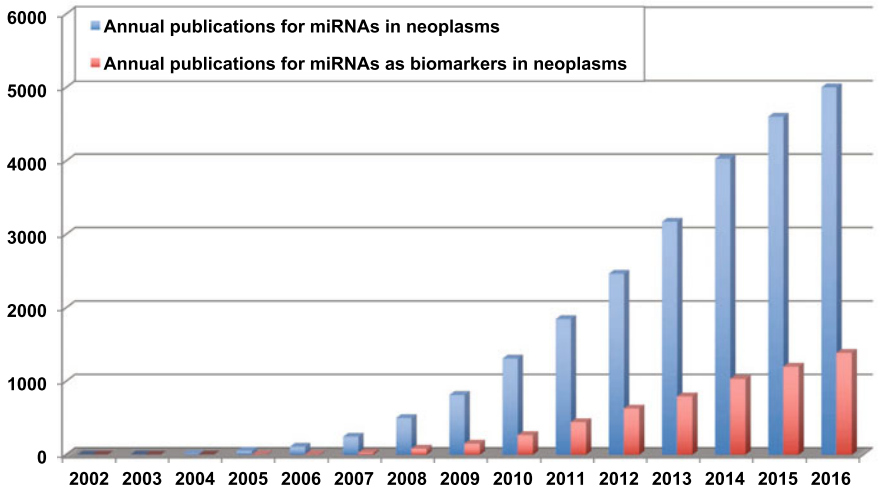

Fig. 1 Total annual miRNA publications indexed in the PubMed database relating to neoplasms and the use of miRNAs as biomarkers. The literature search was performed for the period from 2002 to 2016 using the mesh-terms "microRNA," "neoplasm," and "biomarkers."

identification of miRNAs as potential cancer biomarkers (Fig. 1). More than 6000 papers associated with miRNA cancer biomarkers have been published so far (assessed June 2017). However, despite of this massive number of publications, no circulating miRNA cancer biomarker has yet been implemented in the clinic, thereby emphasizing several challenges within this field of research.

5 Circulating miRNAs as Direct Markers of Neoplastic Growth or of General Disease States

The rationale for identifying circulating miRNAs is often based on the assumption that the miRNAs escape from the primary tumor and are released into the blood stream or other biofluids. They are thereby seen as direct markers of the neoplastic growth. This notion is, however, associated with some conceptual problems. First of all, miRNAs down-regulated in the primary tumor cannot be directly detected in circulation. The tumor has to affect the miRNA expression negatively in other cells in order for the miRNA to be down-regulated in circulation. A decrease in the expression of circulating miRNAs is therefore most likely not a result of the altered expression in the tumor, but a systemic response to the presence of neoplastic growth. With regard to miRNAs up-regulated in the primary tumor, it is very likely that the quantity of this miRNA also increases in the circulation, due to an escape from the cancer cells. However, detecting this increase may be challenging. A recent study made a theoretical calculation of the amount of miRNA that had to be released from a breast cancer tumor compared to normal breast tissue, in order to

measure the increase of the tumor miRNA in the circulation. This study concluded that a stage 1 tumor of 0.5 cm had to release 50,000-fold more RNA into the circulation than the healthy tissue in order to detect a twofold increase of the hypothetical tumor miRNA. The overall conclusion from this study was that circulating miRNAs identified to be associated with cancer (both up- and down-regulated) are more likely a response or a result of a response to the neoplastic growth and not due to altered expression in the tumor itself (Witwer 2015). Another review comparing 154 circulating miRNAs associated with cancer found that only 29% of the miRNAs had the same direction of change in both the primary tumor and in the circulation. This further emphasizes the importance of distinguishing circulating miRNAs as being direct markers of the neoplastic growth or rather correlating with a general disease state (Jarry et al. 2014). This issue of consideration is important when identifying potential miRNA biomarkers for cancer. Circulating miRNAs associated with cancer are still very useful as biomarkers, one just needs to bear in mind that this miRNA alteration may not be caused by a miRNA expression change in the primary tumor.

6 The Challenge of Specificity for Circulating miRNAs as Cancer Biomarkers

The most frequently reported circulating miRNAs in oncology are those with diagnostic potential (Jarry et al. 2014). These are often presented as future biomarkers for the discrimination between individuals suffering from a specific cancer type and healthy individuals. This provides the prospect of applying these miRNA signatures as minimally invasive diagnostic tests for the detection of specific cancer types. However, many of the identified miRNAs are challenged by specificity, which may be one of the main reasons for the lack of circulating miRNA biomarkers in the clinic so far. A great number of the identified circulating miRNAs, such as miR-141 and miR-21, are not specific for one cancer type, but are identified as biomarkers for numerous cancers. miR-21 was in a meta-analysis identified as a significant circulating biomarker for 16 different cancers (Wu et al. 2015). miR-141 has been identified as a circulating biomarker for at least six different cancer types in numerous studies, including prostate cancer. Yet, several other studies did not find a difference in the level of miR-141 between prostate cancer patients and controls (Witwer 2015), emphasizing the challenge of reproducibility, which is further described below. Moreover, a thorough review identified numerous miRNAs (miR-21, miR-155, miR-16, miR-223, and miR-126) often reported as circulating cancer biomarkers also to be associated with more than ten non-neoplastic conditions (Haider et al. 2014). This raises the question of these miRNAs being “true biomarkers for a specific cancer” or rather, and more possibly,

an indication of general disease states such as an activated immune response (Egidi et al. 2013). This notion is further emphasized by miR-21, which has a high expression in activated T-cells and is implicated in the pathogenesis of several autoimmune and chronic inflammatory disorders (Meisgen et al. 2012; Garo and Murugaiyan 2016). The challenge of specificity also raises the question of what the clinician can conclude from these common miRNA biomarkers—that breast, lung, prostate, or colon cancer is present, or that the patient has developed one of possible ten non-neoplastic conditions?

The lack of specificity emphasizes a major challenge within the field of using circulating miRNAs as cancer biomarkers. The identification and use of a single miRNA as a diagnostic or prognostic biomarker for a specific cancer are most likely not feasible. Instead, a panel of miRNAs is probably required for future biomarkers for the identification of a specific cancer or for the differentiation of subtypes.

7 The Challenge of Reproducibility for Circulating miRNAs as Cancer Biomarkers

The challenge of specificity is interconnected with the challenge of reproducibility. Inconsistent findings between numerous independent analyses of a certain cancer type are a common problem within this field of research. A thorough analysis compared 32 studies identifying circulating miRNAs for breast cancer biomarkers published between 2009 and 2014 (Witwer 2015). This study identified a high number of discordant results, completely outnumbering concordant results. A total of 32 publications identified 143 miRNAs significantly altered in plasma or serum from breast cancer patients compared with healthy controls. Of these, 100 miRNAs were identified in only one publication, and further 25 miRNAs, which were observed in more than one study, had contradictory alterations between the studies. Only ten miRNAs were identified in more than one study with a twofold change in the same direction. Of these, only one miRNA, miR-126, was reported in more than two publications. Additionally, the ten miRNAs were all described in publications from the same institution, indicating the use of the same or similar populations (Witwer 2015). Two other studies have also compared circulating miRNAs reported for some of the most commonly studied malignancies (including breast, prostate, gastric, head and neck, colorectal, and non-small-cell lung cancer (Jarry et al. 2014; Kinoshita et al. 2017). No single miRNA was identified in all studies analyzing each specific cancer type, and several miRNAs (such as miR-21, miR-141, miR-155, and miR-145) were identified in numerous cancer types, again emphasizing the problem of specificity. The problem of concordance between studies of the same cancer type may in part be explained by the lack of standardization of the different procedures when identifying new biomarkers, as discussed further below.

8 Clinical Trials Investigating Circulating miRNAs as Potential Biomarkers in Cancer

Despite the promising nature of miRNAs and a remarkably increasing number of publications identifying potential biomarkers based on circulating miRNAs, the challenges of specificity and reproducibility may be the reason for the lack of clinically applicable circulating miRNAs. This incongruity is also reflected in the contrast between the exceedingly high number of publications identifying potential miRNA biomarkers and the considerably lower number of clinical trials investigating the use of miRNAs as biomarkers. Up until now, a total of 464 clinical trials associated with miRNAs have been registered in 40 different countries. Of these, 163 trials were associated with cancer (listed at the US National Institutes of Health database ClinicalTrials.gov by the keywords "miRNA" or "miRNA and cancer," assessed August 2017). Of the 163 clinical trials, 52 were in phase I–IV of which only three studies aimed at the direct analysis and validation of a previously identified miRNA as a circulating cancer biomarker (Fig. 2) (the remaining 111 studies not in phase I–IV were observational or patient registries). We will focus on these three clinical trials, as they are, in our opinion, the most promising advances with regard to translation into a clinical application. We will not provide a summary of all miRNAs identified so far as potential circulating biomarkers for cancer, due to the above-described challenges of specificity and reproducibility as well as the large number of publications and reviews on specific malignancies or miRNAs.

Of the three clinical trials, one aims at evaluating a 15-miRNA panel detected in the serum/plasma of breast cancer patients with invasive metastatic disease. The miRNA panel is to be evaluated as a predictive marker of hormone resistance/sensitivity after treatment with tamoxifen. The study description does not give any references to the preclinical analyses, in which the 15 miRNAs were identified, although the panel is most likely based upon a study from Maillot et al (Maillot et al. 2009). This clinical trial was, however, to be completed in September 2015. This is neither verified at

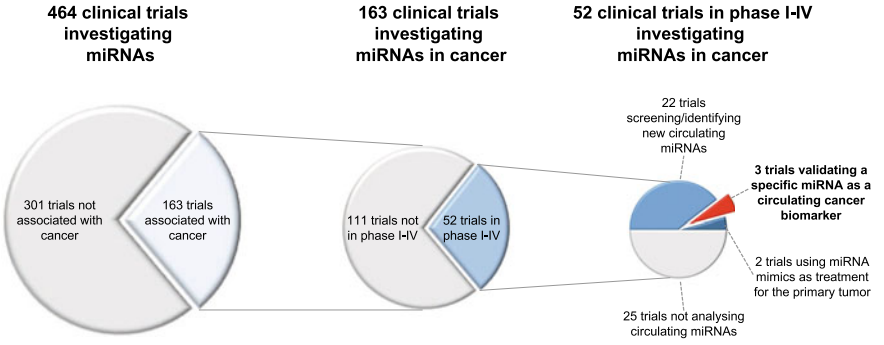

Fig. 2 Clinical trials associated with miRNAs and cancer. The number of clinical trials listed at the US National Institutes of Health database ClinicalTrials.gov by the keywords "miRNA" or "miRNA and cancer" (assessed August 2017)

ClinicalTrials.gov, nor have we been able to find any publications regarding this study. This may be an indication of negative results when validating the miRNA panel as a circulating biomarker.

The second study is a phase III clinical trial registered to investigate the performance of active surveillance in combination with different treatment strategies for pediatric and adult patients with germ cell tumors. This study examines the use of a four-miRNA panel detected in serum as a diagnostic biomarker for malignant germ cell tumors (MGCTs). There is no information regarding the identification of the four miRNAs in the panel, though it is most likely the panel described by Palmer et al (2010). The study was initiated in February 2017 and is scheduled for completion in the end of 2022. Thus, it will be awhile before any results from this study are published.

The third clinical trial is the most promising in regard to the development of a new clinical application and has resulted in the establishment of the company Gensignia Life Sciences Inc. (USA). This study evaluates the use of a 24-miRNA panel as a circulating biomarker for early detection of lung cancer in healthy heavy smokers. This clinical trial is based upon the results from three prior studies, which systematically identified the miRNA panel and validated it in independent patient cohorts (Fig. 3). The first study analyzed two independent cohorts of plasma samples from 19 and 22 lung cancer patients obtained 1–2 years before their diagnosis and at the time of diagnosis (Boeri et al. 2011). They performed a thorough evaluation of the normalization of their data, using two different strategies, as the normalization of miRNA data in plasma samples is still highly debated (the normalization strategies used were (i) determination of the expression ratio between all miRNAs and (ii) normalization to the global mean expression). The two normalization methods identified the same deregulated miRNAs, accentuating their validity. Four different miRNA panels, consisting of 24 miRNAs in total, were identified for the determination of four predictive, diagnostic and prognostic factors, risk of disease, risk of aggressive disease, presence of disease, and presence of aggressive disease (Boeri et al. 2011). In the second study, they combined the four-miRNA prediction panels into one miRNA signature classifier (MSC) for the determination of low, intermediate, or high risk of lung cancer for heavy smokers (Fig. 3) (Sozzi et al. 2014). Several countries have initiated screening trials for the determination of the risk of lung cancer for heavy smokers using a low-dose computed tomography (LDCT). However, this method has a high false-positive rate, indicating a need for complementary biomarkers. They analyzed plasma samples from 870 individuals without lung cancer and 69 with lung cancer. The diagnostic performance of the MSC alone for lung cancer detection was 87% for sensitivity and 81% for specificity. Furthermore, when combining the MSC with the LDCT screening results the false-positive rate was reduced to 3.7% (compared with 19.4% for the LDCT alone). This study clearly indicated that the MSC, consisting of four-miRNA panels combining a total of 24 miRNAs, could be used as a predictive and diagnostic circulating biomarker for the detection of lung cancer in heavy smokers. In the third study, the group then tested the prognostic performance of the MSC by analyzing plasma samples from 84 individuals with lung cancer

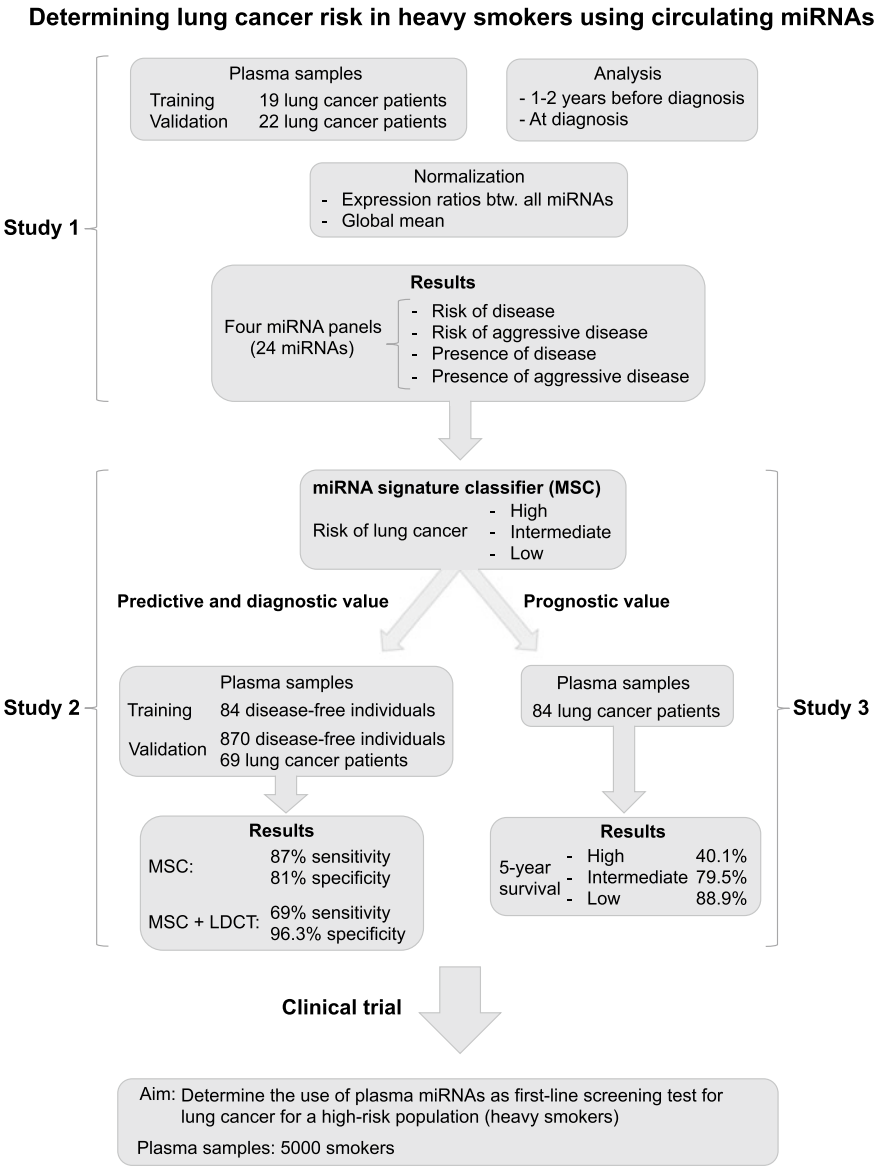

Fig. 3 Outline of three studies identifying a miRNA panel used as a circulating biomarker for lung cancer prediction, detection, and prognosis for heavy smokers. MSC—miRNA signature classifier; btw.—between; LDCT—low-dose computed tomography

(Fig. 3) (Sestini et al. 2015). They identified a significant difference in the five-year survival rates between patients classified as high risk compared with both intermediate and low-risk patients, implying a prognostic value of the MSC as well. In the currently ongoing clinical trial, the MSC will be validated as a first-line

screening test for lung cancer detection (Fig. 3). The miRNA profile will be analyzed in 5000 plasma samples and the risk of lung cancer determined (1000 plasma samples already collected, as well as enrollment of 4000 additional smoking volunteers). The volunteers will be included in a program of active surveillance based on their miRNA risk profile, both in regard to development of lung cancer as well as for the aggressiveness of the disease. The study is registered to have the final data collected in the beginning of 2018. The three prior studies, this clinical trial is based upon, have presented exceptionally encouraging results, providing a positive prospect for the outcome of the clinical trial.

9 Methodological Variables May Have a Vast Impact on Both Specificity and Reproducibility

The progression toward fulfilling the biomarker promise of circulating miRNAs has, as described above, been a work in progress associated with much frustration and many apparently contradictory findings in the literature, resulting in a lack of consistent and robust results. A general trend in the increase of inconsistency of published data has been observed during the last decade and constitutes a matter of concern (McNutt 2014; Bustin 2010). This may in part be explained by the lack of standardization and numerous pitfalls and technical requirements associated with the different processes when identifying new circulating miRNA biomarkers. This includes the pre-analytical phase with sample collection and preparation, the analytical phase determining the miRNA expression levels, and the post-analytical phase handling data extraction and normalization. One of the major challenges when identifying circulating-free miRNAs as biomarkers in cancer is their low amount in biofluids. Hence, even minor alterations in just one of the multiple steps in these procedures may have a great impact on the final miRNA quantification and may lead to potentially biased or uninterpretable results (Table 1).

10 Pre-analytical Challenges

Pre-analytical variables can have a large effect on downstream analyses. These variables can generally be divided into sample source, sample collection and handling, patient factors, and the power of the study. Hence, controlling such variables is therefore of utmost importance for the identification and future use of circulating miRNAs as non-invasive diagnostic and prognostic biomarkers. Currently, no standard documentation exists for these pre-analytical variables and they are not routinely documented in research papers investigating circulating miRNAs, making it challenging to compare studies. We will, in this section, discuss the pre-analytical variables, which may affect the integrity of the biofluid sample, and the significant consequences this may have on the downstream analyses. We have

Table 1 Variables, associated with the pre-analytical, analytical, and post-analytical phases, influencing the final miRNA quantification in blood samples

Pre-analytical phase	Influence on miRNA analysis
Sample collection— Blood samples	
• Method of collection	• The venipuncture site may induce hemolysis from which cellular material is released into the sample specimen
• Time span between sample collection and centrifugation	• The time between collection and centrifugation may correlate with the amount of hemolysis
• Choice of plasma or serum	• Serum: Separation time: <30 min may retain cellular elements; >60 min may facilitate lysis of cells in the clot thereby releasing cellular components into the serum
	• Plasma: The use of different anticoagulants (EDTA, heparin, and sodium citrate) may influence the protein composition and thereby affect downstream analysis
• Hemolysis effect	• All samples need to be validated for hemolysis and how this will affect downstream analyses
• Presence of endogenous inhibitors	• May inhibit the Taq polymerase and thereby lead to false-negative PCR reads
Preparation of samples for analysis	
RNA extraction	
• RNA isolation protocols (such as trizol, bead-based, or column-based)	• miRNA recovery can be largely influenced by the isolation method
• Adjustment of input amount	• As the RNA quantity cannot be measured with a spectrophotometer after extraction, due to the low miRNA levels in biofluids, it is important to use a fixed input volume for all samples in the extraction, to eliminate differences detected due to different amounts of starting material
• Spike-in controls	• Can be used to determine the extraction efficiency for each sample, especially for samples such as biofluids which contain low levels of miRNAs
• Biological and technical replicates	• By making several RNA purifications from the same sample/patient the noise caused by variation is minimized. Especially important for samples containing low levels of miRNAs, such as biofluids
RNA quality control	
• Purity assessment (absorption ratios 260:280 nm and 230:260 nm)	• Contaminants may inhibit PCR reactions, thereby causing false-negative results

(continued)

Table 1 (continued)

Pre-analytical phase	Influence on miRNA analysis
cDNA synthesis	
• Replicates	• Both biological and technical replicates are important to minimize the noise caused by variations due to the RT enzyme
• Negative RT control	• If not all genomic DNA has been removed, this may influence downstream miRNA analyses
• Positive controls (Spike-ins)	• Indicates the presence of inhibitors
• Sufficient details regarding the setup	• Information regarding primer sequences/assay IDs, reagents, concentrations, reaction conditions, and instrument used will help facilitate comparability between studies
Analytical phase	
Choice of miRNA detection platform	• Both sensitivity and specificity are influenced by the method of choice
• qPCR-based technologies (miRCury (Exiqon), TaqMan (Qiagen), OpenArray (Thermo Fisher Scientific), qScript (Quanta BioSciences), and SmartChip (WaferGen))	
• Hybridization platform (microarray (Thermo Fisher Scientific)	
• Sequencing (TruSeq (Illumina), Ion Torrent (Thermo Fisher Scientific))	
Post-analytical phase	
Data normalization	
• Larger screening studies	• Carefully evaluate and select the most appropriate method based on the given experimental setup
• Studies analyzing a limited number of miRNAs	• The number and choice of reference miRNAs should be carefully evaluated in the given dataset
• Reporting raw data	• Will help facilitate comparability between studies

chosen to focus on the use of blood (serum and plasma) as source of the analyzed miRNAs. However, most of the variables described are also relevant for other sources of biofluids.

10.1 Blood Samples—Choice of Plasma or Serum

Using blood for the detection of circulating miRNAs is an obvious source, as the collection is minimally invasive and blood samples are taken routinely in the clinics

for the treatment and follow-up processes of most cancers. Furthermore, as the sample collection is minimally invasive, it allows repeated sampling and can be used as a prognostic tool for monitoring disease state over a course of time. However, several critical points need to be taken into consideration when analyzing circulating miRNAs in blood samples. The venipuncture site itself may induce hemolysis and thereby contamination by platelet derived miRNAs (Lippi et al. 2012; Lance et al. 2013). The elapsed time between blood collection and processing is also of crucial importance as this may influence the amount of lysis and thereby cellular contamination (Ayers et al. 2011). Furthermore, for the detection of circulating miRNAs in blood samples, it is important to determine whether to use plasma or serum as the source. One study, comparing 154 publications analyzing circulating miRNAs in blood samples, found an almost equal distribution between the use of plasma or serum. However, a disadvantage of using serum rather than plasma is the release of platelet miRNAs into the serum during clot formation (Witwer 2013; Gemmell et al. 1993). The time for clot formation for the serum sample may also influence the amount of circulating-free miRNAs and downstream analyses. If less than 30 min are allowed for clot formation, cellular elements and other contaminating factors are likely to be retained in the specimen, impacting downstream analyses, whereas more than 60 min of clot formation may induce hemolysis of cells in the clot (Tuck et al. 2009). Release of miRNAs from blood cells will greatly alter the measured miRNA profile with ~2/3 of miRNAs detected in the plasma potentially affected by hemolysis (Kirschner et al. 2013). Numerous circulating-free miRNAs identified as potential biomarkers for cancer have been associated with hemolysis of erythrocytes (e.g., miR-21 and miR-16) (Kirschner et al. 2013; Shkurnikov et al. 2016). It is particularly important to avoid reference miRNAs used for normalization (such as the frequently used miR-16), being influenced by hemolysis, as any changes in the level of these miRNAs, may have a large impact on the total miRNA concentration detected. As hemolysis was detected in over 40% of analyzed clinical specimens (Hawkins 2010), it is of utmost importance to thoroughly examine the effect of hemolysis for all circulating-free miRNAs and exclude the hemolysis susceptible miRNAs. Several methods to detect hemolysis have recently been compared demonstrating a high sensitivity of the ratio of the red blood cell-enriched miR-451a compared to the reference miRNA miR-23a-3p for detecting hemolysis (Shah et al. 2016; Blondal et al. 2013). Although the use of plasma may eliminate some of the problems associated with hemolysis, a drawback of using plasma is, however, the need for anticoagulants, such as heparin and EDTA. Both, but particularly heparin, are associated with false-negative PCR amplifications, due to inhibition of the Taq polymerase (Huggett et al. 2008; Beutler et al. 1990; Yokota et al. 1999).

Most studies analyzing circulating miRNAs in blood have used whole plasma and serum, and not purified protein/miRNA-complexes (Larrea 2016), indicating a need for further investigations of the differences between plasma and serum and how they are affected by sample processing. Even small changes in the miRNA expression, due to variables associated with sample collection and preparation, may

have a great effect on downstream analyses, due to the low concentration of miRNAs in biofluids.

10.2 Patient Factors and Power of the Study

Apart from the technical variables described above, inter-individual differences may also impact and bias the interpretation and conclusion of studies identifying circulating biomarkers in cancer. Both individual variability (such as sex, ethnicity, and age) and external differences and life-styles (such as smoking habits, diet, drug assumption, different chemotherapy treatments, and physical activity.) may influence the miRNA expression level in circulation (Tiberio et al. 2015). Some of these variables (such as sex, ethnicity, and age) can be adjusted for in the patient selection. However, most of the external differences are difficult to evaluate and to take into consideration when selecting the patient and control groups. Hence, when evaluating newly identified circulating miRNA cancer biomarkers, it is important to discuss the possibility of these being identified due to differences in individual behavior rather than differences in disease states (Tiberio et al. 2015). Therefore, besides the careful selection of a homogenous patient group, it is important to calculate the power of the study, in order to determine the number of samples needed for both case and control groups to possibly eliminate some of the external differences between individuals and detect the true effects the study/hypothesis aims at. Insufficient power may also be part of the explanation for the lack of reproducibility between studies (Jarry et al. 2014).

11 Preparation of Samples for Analyses

11.1 Extraction Methods and Quality Control

The method used for extraction of the miRNAs from the biofluid sample can also influence the final result and the miRNAs identified as future biomarkers. Numerous different protocols and commercially ready-to-use kits are available for miRNA extraction from biofluids. This contributes to a large diversity between studies and makes it difficult to compare the identified circulating miRNAs. This challenge has been particularly emphasized by several studies comparing different extraction methods, which determined the extraction method to be a major factor of variability for the miRNA analysis and poor recovery rates were obtained for many of the protocols evaluated (Ralla et al. 2014; El-Khoury et al. 2016; Brunet-Vega et al. 2015). To determine the recovery rate for a selected purification method, it is recommended to spike the biofluid sample with synthetic, non-human miRNAs (such as the *C. elegans* cel-miR-39 and cel-miR-238). This is particularly important when analyzing circulating-free miRNAs, as they are present at very low levels, and therefore even minor differences in the extraction efficiency may have a major

impact on downstream analyses. The low levels of miRNAs in biofluids also impair quantification of the extracted RNA using a spectrophotometer. It is therefore important to use a fixed volume of input material for all samples in the extraction to eliminate differences detected downstream, as a result of different amounts of starting material (Tiberio et al. 2015). The amount of starting material may also impact the choice of extraction method, as the performance of kits has been shown to vary when using low and high sample volumes (El-Khoury et al. 2016). It is therefore important to consider the type and size of input material and carefully select the RNA isolation method in order to avoid biased results (El-Khoury et al. 2016). The use of both biological and technical replicates can help minimize noise caused by variations due to the selected extraction method.

During the miRNA extraction, potential PCR inhibitors may be isolated together with the miRNAs. It is therefore important to determine the purity of the extracted miRNA, by measuring the 260/280 nm ratio for protein contamination and the 260/230 nm ratio for other contaminants (such as guanidine salts and phenol).

11.2 cDNA Synthesis

The reverse transcription (RT) of (mi)RNA to cDNA is a fundamental step in miRNA expression analyses. However, the efficiency of the RT enzyme has been shown to be highly variable and introduce significant variation in downstream RT-qPCR analyses (Bustin et al. 2009, 2015). There are several different cDNA kits available (e.g., from ThermoFisher Scientific and Exiqon), which may perform differently with regards to RT efficiency, as it has been shown for cDNA synthesis for mRNA analysis (Bustin et al. 2015). There has, however, to our knowledge, not been any thorough comparison between cDNA kits for miRNA analysis so far. It should therefore be kept in mind that these kits may have different performances, which could influence downstream analyses. Thus, apart from detailed information of the protocol used, it is important to determine and minimize the variability by using multiple RT replicates for each sample, whenever possible. Also, the amount of input material for the reverse transcription should be standardized. However, as mentioned above, this is impractical when analyzing biofluid samples, and should instead be standardized in the RNA purification step. A negative RT control (with no RT enzyme in the reaction) should be included in order to determine the presence of contaminating DNA, which may influence downstream analyses. Positive controls (e.g., synthetic spike-ins) should be included to determine the presence of inhibitors.

The above-mentioned pre-analytical considerations demonstrate the importance of understanding and standardizing these variables and report all analysis details in order to enable comparisons of different miRNA studies and obtain reproducible results.

12 Analytical Challenges

12.1 Applications for miRNA Detection in Biofluids

After collection and preparation of the samples, the expression profile of the circulating miRNAs is measured. Mature miRNAs have a very small size of only 22–25 nucleotides and there is a high degree of homology between miRNA family members. These two factors, combined with the low amount of circulating-free miRNAs in biofluids, make it technically challenging to determine the expression profile of circulating miRNAs. Nevertheless, several platforms have been developed for the quantification of miRNA expression. These platforms use different approaches for miRNA detection, such as qPCR-based technologies, hybridization, and sequencing. A study by Mestdagh et al. compared and evaluated nine different miRNA expression platforms for serum sample analysis (Mestdagh et al. 2014). They evaluated six different qPCR technologies [miRCury (Exiqon), OpenArray (Thermo Fisher Scientific), TaqMan Cards preAmp (Thermo Fisher Scientific), miScript (Qiagen), qScript (Quanta BioSciences), and SmartChip (WaferGen)], one hybridization platform [microarray (Agilent)] and two sequencing technologies [TruSeq (Illumina), and Ion Torrent (Thermo Fisher Scientific)]. This study showed a vast difference in sensitivity between the different technologies, with qPCR platforms performing superior in regard to sensitivity, whereas the hybridization and sequencing platforms had a poor sensitivity when analyzing serum samples. Furthermore, the qPCR platforms also showed a high accuracy, which, combined with a strong sensitivity, gives a very reliable quantitative measurement. This study concluded that out of the nine platforms analyzed, four of the qPCR-based technologies [miRCury (Exiqon), miScript (Qiagen), qScript (Quanta BioSciences), and TaqMan Cards preAmp (Thermo Fisher Scientific)] had a superior performance in regard to both sensitivity and accuracy when analyzing serum samples. Furthermore, when evaluating differential miRNA expression, they observed substantial inter-platform differences. This may have an immense impact on studies using the same method for both screening and validation. The study therefore recommended the use of two different platforms or technologies for screening and validation analyses, to eliminate biased results due to the method of choice (Mestdagh et al. 2014).

13 Post-analytical Challenges

After selection of the optimal detection method, the next question is how the raw data should be processed. A critical step in the processing of the raw data is normalization. Systematic differences caused by variation in the experimental processes can introduce data artifacts. These should be carefully removed to make the data comparable between samples and only identify truly biologically deregulated miRNAs in subsequent analyses. However, numerous normalization strategies

are available and no consensus exists in regard to the most optimal method (Jarry et al. 2014; Schwarzenbach et al. 2015).

In larger screening studies (>100 miRNAs analyzed), commonly used normalization methods for gene expression microarrays are often used (Zhao et al. 2010; Qin et al. 2013; Meyer et al. 2012). These methods are based on the assumption that a large proportion of the targets analyzed are not differentially expressed. This assumption may, however, not hold true for miRNA screening studies. The number of miRNAs in the genome is approximately tenfold less than mRNA species and in most studies detection is limited to only a few hundred miRNAs, of which a large proportion is often differentially expressed (Qin et al. 2013). Several studies have evaluated the most appropriate normalization method for miRNA screening studies, but concluded on different optimal normalization strategies. However, they have assessed different setups and platforms, which may explain the different normalization methods identified as the best performing (Zhao et al. 2010; Qin et al. 2013; Meyer et al. 2012). New normalization methods developed directly for miRNA data would most likely be the most optimal solution for these analyses. However, until this exists, researchers need to carefully evaluate the assumptions for each normalization method and select the most appropriate for their experimental settings (Zhao et al. 2010).

In validation studies, in which only a limited number of miRNAs are analyzed, the normalization is often done using the expression level of either small nucleolar RNAs (snoRNAs), a synthetic spike-in, a single miRNA, or a subset of miRNAs (Jarry et al. 2014). In order to be a suitable for normalization, the selected reference (s) should be stably expressed within the given dataset and their abundance should have a strong correlation with the total amount of miRNA present in the samples (Bustin et al. 2009). As advised by the MIQE-guidelines, the number and choice of reference genes must be carefully evaluated and experimentally determined for each specific sample cohort being studied (Bustin et al. 2009). We strongly advise not to use synthetic spike-ins to normalize against. These can be used for standardization, when correcting for differences in RNA recovery and qPCR efficiencies, but not for differences in endogenous miRNA expression between samples (Jarry et al. 2014). The use of both snoRNAs or single miRNAs for normalization, without validation of their expression in the given dataset, is often done in the belief that their expression is independent of health status (Jarry et al. 2014). This has, however, been shown numerous times to be invalid, as no snoRNAs or single miRNA have been identified, which is not implicated in any disease states (Jarry et al. 2014). It is therefore important to carefully select the most stably expressed miRNAs in the given dataset and also determine the optimal number of miRNAs used for the normalization.

As no consensus exists in regard to the most ideal normalization method, it is very important for any miRNA screening study to report all information regarding the experimental setup and make the raw data publicly available, in order for other researchers to compare previous studies with their own studies. This may help minimizing the challenge of reproducibility.

14 Conclusion

Despite the promising nature of circulating miRNAs as cancer biomarkers and the vast number of publications identifying potential circulating miRNAs as both diagnostic and prognostic biomarkers for numerous cancers, no specific circulating miRNA or panel of miRNAs has yet been implemented in the clinic. The comparability between the current studies is limited, due to discrepancies in both the pre-analytical, analytical, and post-analytical stages. A more profound focus on optimization and standardization of the different procedures when identifying circulating miRNAs as cancer biomarkers may help overcome the challenges of specificity and reproducibility, enabling a successful translation of basic research into the clinics.

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Circulating MicroRNAs as Potential Biomarkers for Lung Cancer

Sabrina Müller, Florian Janke, Steffen Dietz and Holger Sültmann

1 Introduction

1.1 Lung Cancer

Lung cancer is one of the most frequently diagnosed types of cancer and the leading cause of malignancy-related death (Ferlay et al. 2015). Due to the absence of clinical symptoms in the early phases of the disease and the lack of effective screening programs, lung cancer is commonly diagnosed in advanced stages (Goldstraw et al. 2016; Nicholson et al. 2016). Tumors of the lung can be classified into two major types, i.e., small cell (SCLC; 15%) and non-small cell (NSCLC; 85%). The latter can be further divided into three main subtypes: adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large-cell carcinoma (LCC). Each subtype has a unique growth pattern and genetic disposition (Herbst et al. 2008). However, mixed histological subtypes of NSCLC have also been observed (Sakashita et al. 2014). The therapy of lung cancer patients is based on the tumor type, the TNM stage, and the presence of targetable mutations. The prognosis depends mainly on the staging of the tumors (Travis et al. 2013): The overall five-year survival rate for lung cancer is only about 19.5%, mainly due to late diagnosis. When diagnosed at an early local stage, the five-year survival rate increases to 50% or higher. In contrast, for patients with metastasized tumors, it drops to 1% (Siegel et al. 2018).

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Screening with chest X-ray or low-dose CT has been shown to detect lung cancer earlier (Chin et al. 2015). However, these procedures bear the risk of harm due to radiation, overdiagnosis, and false-positive results (ten Haaf et al. 2017). Thus, there is still an urgent need for novel biomarkers for early detection, to increase the survival rates of patients suffering from lung cancer. In recent years, the potential of microRNAs (miRNAs) as lung cancer biomarkers has been tested extensively. Here, we summarize the current status of these studies and discuss the utility of miRNA biomarkers in the blood of NSCLC patients.

1.2 miRNA Biogenesis and Biological Functions

miRNAs are part of a family of small non-coding RNAs (20–22 nucleotides) that regulate a broad range of biological processes. The biogenesis of miRNAs starts with the synthesis of pri-miRNAs by RNA polymerase II (Fig. 1; Borchert et al. 2006). Subsequently, pri-miRNAs bind to a microprocessor complex of Drosha and DGCR8, where they are processed into precursor miRNAs (pre-miRNAs), which are about 85 nucleotides in length and have a stem-loop structure. These pre-miRNAs are exported from the nucleus into the cytoplasm by Exportin-5 (MacFarlane and Murphy 2010). There, they are further cleaved by the Dicer protein into miRNA duplices, and the strand complementary to the single-stranded mature miRNA is degraded. Upon binding of the mature miRNA to the Argonaute 2 (AGO2) protein, the RNA-induced silencing complex (RISC) is formed. The miRNA serves as a guide directing RISC to the target mRNA (Bartel 2009), which leads either to degradation and cleavage of the mRNA molecule through perfect complementary base pairing, or to a inhibition of protein translation through an imperfect complementary base pairing (Krol et al. 2010; Vasudevan et al. 2007). Since binding to mRNA does not have to be absolutely complementary, one miRNA can regulate many different mRNAs. Similarly, one mRNA molecule can be regulated by several different miRNAs (Hayes et al. 2014).

To date, there are 2654 human miRNAs registered in miRBase22 (www.mirbase.org), a database encompassing published miRNAs and their annotations. Many of these are evolutionarily highly conserved, highlighting their importance in controlling cellular processes (Lu et al. 2005; Lewis et al. 2005) such as cell division, differentiation, and apoptosis (Hayes et al. 2014; Li et al. 2012). It is estimated that at least 50% of all protein-coding genes are regulated by miRNAs (Fromm et al. 2015). Consequently, altered expression of miRNAs can be found in almost any type of cancer cell (Peng and Croce 2016). Like other transcripts, miRNAs are also subject to genomic changes, e.g., amplifications, deletions, and translocations (Peng and Croce 2016). Furthermore, it has been shown that miRNA expression is modulated by DNA hyper- and hypomethylation (Han et al. 2007; Saito and Jones 2006). Vice versa, oncogenic processes including aberrant methylation of promoter regions and rearrangement of chromosomal regions, are affected by deregulated miRNAs (Lujambio et al. 2008; Calin et al. 2004; He et al. 2007).

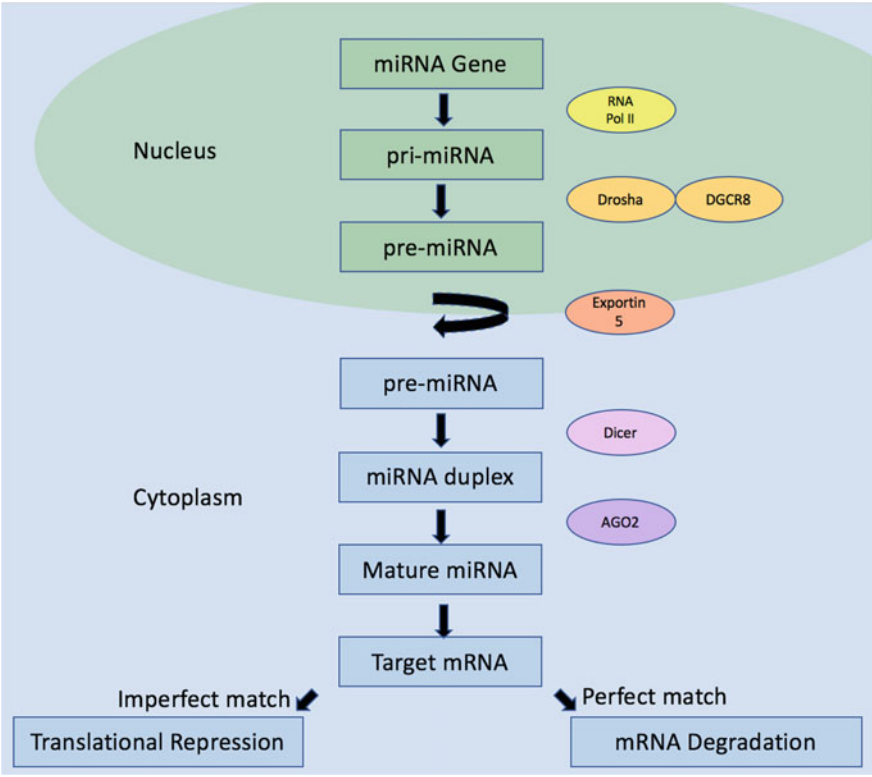

Fig. 1 miRNA biogenesis

1.3 miRNAs as Blood-Based ("Liquid Biopsy") Biomarkers in Cancer

miRNAs are the most abundant type of cell-free RNA (cfRNA) found in the blood (Siravegna et al. 2017). They can also be contained in exosomes, apoptotic bodies, protein miRNA complexes (Vickers et al. 2011), or tumor-educated platelets (Joosse and Pantel 2015). Their stability in body fluids makes miRNAs suitable candidates as biomarkers in oncology. They are resistant to extreme pH and temperatures, as well as against degradation by RNases (Schwarzenbach et al. 2011). They can withstand repeated freeze/thaw cycles (Mitchell et al. 2008). miRNAs in various body fluids, such as cerebrospinal fluid (Gui et al. 2015), pleural effusion (Ak et al. 2015), urine (Urquidi et al. 2016), breast milk (Do Canto et al. 2016), saliva (Ding et al. 2016), blood (Zhang et al. 2017), but also in breath condensate (Khalil et al. 2017), have been shown to be useful biomarkers. In this way, they can provide information about pathological processes in the organism (Chen et al. 2008). The abundance of miRNAs in the blood correlates with that of their tumors of origin (Brase et al. 2011). This has led to the exploration of the utility of

miRNAs as potential non-invasive biomarkers for cancer diagnosis (Mitchell et al. 2008). Different abundances of circulating miRNAs between many types of cancer and healthy subjects (Ohtsuka et al. 2015; Schwarzenbach et al. 2011), but also in non-malignant diseases such as Alzheimer's or Parkinson's disease (Butz et al. 2016), have been reported.

2 Technologies for Processing and Analysis of Circulating MicroRNAs

A major advantage of serum and plasma over whole blood is the possibility of long-term storage at temperatures of −80 °C or below without significant reduction of sample quality (Kirschner et al. 2011). Therefore, serum and plasma are largely favored as starting materials. Importantly, blood collection and handling should be performed following standardized guidelines aiming to reduce the contamination with miRNAs from other sources like lysed red blood cells (hemolysis) or leukocytes in order to reach optimal performance and comparability among studies (Kannan and Atreya 2010).

Due to the low amount of miRNA in body fluids, efficient miRNA extraction methods are required (Fig. 2). Prior to miRNA extraction, non-human miRNAs are often added as "spike-ins" to the sample. The recovery of these "spike-ins" is then used as a measure for the variation of extraction efficiencies between samples (McDonald et al. 2011). Commercially available miRNA extraction kits that combine organic extraction with silica-membrane-based purification have shown to yield better results in terms of protein removal and therefore replaced pure Trizol® (Phenol:Chloroform) extraction in many laboratories (Kim et al. 2012a). Also, kits utilizing non-Trizol® sample homogenization followed by silica-membrane-based miRNA extraction are commercially available and have shown good performance (Baggish et al. 2011; Eldh et al. 2012). Trizol®-based extraction has its advantages in the large range of starting volumes (Hu et al. 2010; Corsten et al. 2010) as well as its higher efficiency in isolating miRNAs from extracellular vesicles (Moldovan et al. 2013; Eldh et al. 2012). Silica-membrane-based extraction techniques require shorter turnaround times (Hunter et al. 2008) and allow a more efficient isolation of small RNA molecules with low GC content, which are often selectively lost in Trizol®-based extraction (Kim et al. 2012b).

To assess miRNA quality, the size distribution of the obtained small RNA fragments is routinely checked using highly sensitive capillary electrophoresis (Jung et al. 2010). By integration of the small RNA peak in the electropherogram, the miRNA quantity can also be estimated. Using fixed amounts of starting material has shown to result in improved reproducibility when compared to equal miRNA concentrations (Mitchell et al. 2008; Kroh et al. 2010). Another frequently performed quality control is to determine the degree of hemolysis in the sample as an indicator for appropriate sampling (Appierto et al. 2014). Ideally, this is done as a pre-analytical quality check by spectrophotometry and/or post-analytically using

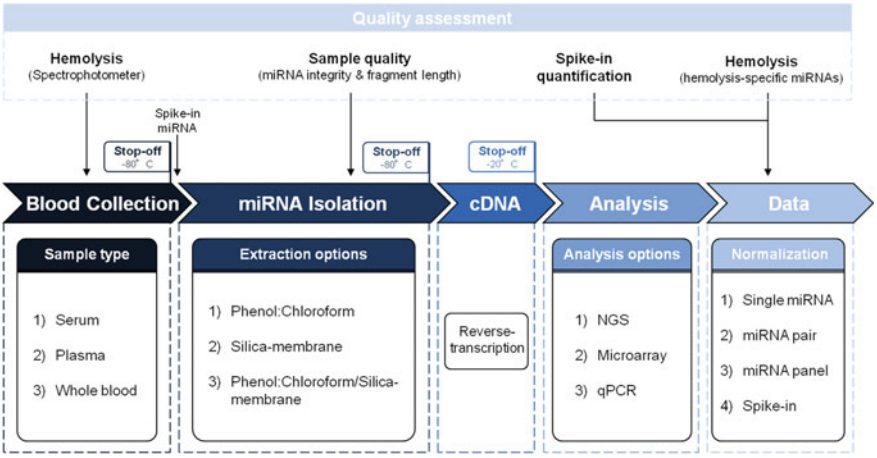

Fig. 2 Circulating miRNA abundance analysis workflow

one or several hemolysis-specific miRNAs (e.g., *miR-451*) as markers (Fortunato et al. 2014; Shah et al. 2016).

For the quantification of miRNAs of interest, several options are available all of which require a previous reverse transcription step to generate cDNA from the miRNA template. Currently, the methods most commonly used for miRNA abundance analysis are qRT-PCR, microarrays, and next-generation sequencing (NGS) (Tiberio et al. 2015). In terms of simplicity, as well as sensitivity and specificity, qRT-PCR is the most widely used method. It offers a relatively cost-efficient possibility of analyzing several miRNAs with straightforward data processing and interpretation (Jensen et al. 2011). However, if the experiment aims at simultaneously analyzing many miRNAs, microarray analysis is widely used for biomarker screening using a subset of already described miRNAs. NGS is the only analysis method with the potential of detecting yet undiscovered miRNAs (Pritchard et al. 2012).

A major challenge in processing miRNA analysis data is the normalization of the obtained miRNA abundances (Schwarzenbach et al. 2015). Housekeeping genes such as RNU48 or RNU6, which are often used in tissue, cannot be reliably determined in liquid samples, as they are—due to RNase-mediated degradation—not always detectable in the circulation (Wang et al. 2012). High-throughput methods including microarrays and NGS benefit from the large number of analyzed miRNAs. Here, approaches like quantile normalization can be applied, which consider all measured miRNAs for the normalization (Mestdagh et al. 2009). In qRT-PCR, the search for miRNAs with a high and rather invariant abundance across various tissues is ongoing. Algorithms like geNorm, NormFinder, and Bestkeeper have been employed to identify such miRNAs. However, normalizer miRNAs vary greatly between studies (Schwarzenbach et al. 2015). Single miRNAs [e.g., *miR-16* (Müller et al. 2014) and *miR-191* (Peltier and Latham 2008)],

miRNA pairs [e.g., *miR-16/miR-93* (Song et al. 2012)], miRNA panels (Bianchi et al. 2011), and spike-in miRNA [e.g., *cel-miR-39* (Wang et al. 2010)] have been used in different studies to normalize miRNA abundances. In summary, several methods for miRNA isolation and subsequent analysis are readily established and commercially available, making miRNA analysis in liquid biopsy samples easily applicable.

3 Circulating MicroRNA as Biomarkers in Lung Cancer

Many studies have shown the applicability of circulating miRNAs as biomarkers in NSCLC patients. Aberrant circulating miRNA levels are able to discriminate NSCLC patients from healthy individuals [diagnosis (Zhang et al. 2017; Hu et al. 2016)]. In addition, distinct miRNAs were associated with patient outcome [prognosis (Yanaihara et al. 2006; Chen et al. 2013)] and treatment response [prediction (Zhu et al. 2016a; Franchina et al. 2014)]. Although several studies have focused on individual miRNAs as cancer biomarkers, these are often inferior in terms of sensitivity and specificity when compared to combinations of multiple miRNAs (miRNA panels). This was shown in various meta-analyses reporting up to 9% sensitivity and specificity increase, when miRNA panels were compared to single miRNAs (Wu et al. 2014; He et al. 2015; Wang et al. 2015a). Thus, individual miRNAs may not comprehensively reflect the complex processes involved in carcinogenesis, limiting their efficiency as biomarkers (Wang et al. 2016).

3.1 Diagnostic Circulating MicroRNAs in Lung Cancer

The average five-year survival rate of lung cancer patients drops from approximately 49% in stage I to 1% in stage IV (Siegel et al. 2018). This is the main reason for the importance of biomarkers for early diagnosis. Consequently, many studies have aimed at identifying valid miRNA markers to detect lung cancer as early as possible (Table 1). *MiR-21* is one of the most abundant and recurring oncogenic miRNAs, since it is found to be overexpressed in almost every type of solid cancer. It modulates tumorigenesis by inhibiting negative regulators of the *RAS/MEK/ERK* pathway. *MiR-21* also silences *PTEN* (Zhang et al. 2010), *PDCD4* and *TPM1*, thereby prolonging cell proliferation and migration while inhibiting apoptosis (Zhu et al. 2008). In a study encompassing 40 NSCLC subjects (23 patients, 17 controls), a significant difference in the abundance of *miR-21* was able to distinguish the two groups with 70% sensitivity and 100% specificity. Furthermore, it was shown that *miR-21* abundance was not confounded with age, localization, pack years, staging, or tumor histology (Xie et al. 2011).

Efforts to improve diagnostic procedures have led to the concept of diagnostic miRNA panels in order to maximize prediction efficiency: A panel comprising 34 miRNAs for early detection of NSCLC in a population of asymptomatic high-risk

Table 1 Selected circulating miRNAs distinguishing NSCLC patients from healthy controls

miRNA	Expression level of target miRNA	Subtype differentiation	Starting material	References
miR-17	Overexpression	No	Plasma	Bianchi et al. (2011)
miR-21	Overexpression	No	Plasma, sputum	Li et al. (2011), Shen et al. (2011)
miR-126	Down-regulation	No	Plasma, sputum	Bianchi et al. (2011)
miR-141	Overexpression	No	Plasma	Nadal et al. (2015)
miR-183	Overexpression	No	Plasma, sputum	Zhu et al. (2016)
miR-200	Overexpression	No	Plasma, sputum	Yu et al. (2010)
miR-205	Overexpression	Yes	Tissue, plasma	Aharonov et al. (2009), Leng et al. (2017)
miR-210	Overexpression	No	Plasma, sputum	Zhu et al. (2016)
miR-375	Overexpression	Yes	Tissue, plasma	Yu et al. (2010), Nishikawa et al. (2011)
miR-486	Down-regulation	No	Plasma, sputum	Bianchi et al. (2011), Yu et al. (2010)

patients could distinguish between benign and malignant lesions (Bianchi et al. 2011): The sample selection was based on low-dose CT screening for high-risk patients in the COSMOS study (Veronesi et al. 2008). The 59 asymptomatic patients that had developed a malignant nodule were compared with 69 healthy test subjects. From a group of 365 miRNAs (among them *let-7a*, *miR17-92* cluster, *miR-126*, and *miR-486*), the panel was able to differentiate the serum samples of healthy subjects and lung cancer patients with a sensitivity of 80%. Since the selected subjects were high-risk asymptomatic patients, its intention was to find miRNAs suitable for early detection. However, the study showed that the same 34-miRNA panel was able to detect lung cancer also in later stages (Bianchi et al. 2011). The *miR17-92* cluster consists of seven miRNAs including *miR-17*, *miR-19*, *miR-20a,* and *miR-92a* and is overexpressed in lung cancer. This overexpression leads to down-regulation of *E2F1*, *HIF1A,* and *PTEN*, promoting cell proliferation and cancer progression (Osada and Takahashi 2011).

A similar study found that in the sputum of 122 individuals (64 cancer patients and 58 healthy controls), a panel of four miRNAs (*miR-21*, *miR-468*, *miR-375*, *miR-200b*) distinguished patients from healthy individuals with 81% sensitivity and 92% specificity (Yu et al. 2010). *MiR-200* is a miRNA family consisting of the *miR-200a*, *miR-200b*, *miR-200c* and *miR-429*, which play important roles in the promotion of epithelial to mesenchymal transition (EMT). The down-regulation of the *miR-200* family via ZEB (zinc finger E-box-binding homeobox) transcription factors (*ZEB1* and *ZEB2*) leads to E-cadherin and vimentin overexpression and subsequent progression of lung cancer (Takeyama et al. 2010).

In order to find a detection method for the lowest possible (and therefore best treatable) tumor stage, another study (Shen et al. 2011) examined samples from patients with solid pulmonary nodules (SPN) who were detected by CT scanning, but had not developed symptoms. Thirty-three patients with benign SPN, 32 with malignant SPN, and 29 heavy smokers were recruited. Plasma samples of the subjects were tested using a panel of five miRNAs (*miR-21*, *miR-126*, *miR-210*, *miR-375*, and *miR-486*), which was developed based on their expression profiles in tissue samples. While *miR-126* and *miR-375* showed no differences, the three remaining miRNAs enabled accurate differentiation between the patients (sensitivity 86%, specificity 97%). Since the panel was able to identify stage I lung cancer patients, it might be suitable for early detection of lung cancer in blood samples (Shen et al. 2011).

Even though many miRNA-based detection panels currently exist, none have so far been included in standard medical practice. A first attempt toward exploiting miRNA abundance in liquids was the MILD trial (Tokumaru et al. 2008), where 939 patients were tested using a panel of 24 miRNAs (including *miR-16*, *miR-133a*, cluster 17–92, and *miR-126*) to combine their testing with the result of low-dose CT screenings. The aim was to use them to detect lung cancer as well as to predict the prognosis and the likelihood of death. The data suggested that it was possible to lower the rate of false-positive results from the low-dose CT from 19.4 to 3.7%. The 24 miRNAs were divided into different subtypes but the miRNA panel was unable to further divide the tumors into clinically relevant subcategories (Sozzi et al. 2014).

3.2 Prognostic Circulating MicroRNAs in Lung Cancer

Patients with similar tumor stage can vary considerably in their prognosis, depending on tumor type, histological subtype, as well as molecular changes. In order to improve clinical decision making, many studies have identified prognostic miRNA biomarkers (Table 2).

MiR-21 (up-regulated in NSCLC) and *let-7* (down-regulated in NSCLC) have been shown to be the most useful markers associated with patient outcome. A study using 56 NSCLC patient samples was able to identify a panel (consisting of *let-7a*, *miR-221*, *miR-137*, *miR-372,* and *miR-182*) for prognostic classification. This panel was confirmed with a test set of 62 NSCLC patients. Furthermore, an independent cohort study with another 62 patients was able to classify the prognosis for these patients, regardless of staging or tumor histology (Yu et al. 2008). Another study with serum samples from 391 patients investigated the use of miRNAs targeting genes in the *TGF*-β signaling pathway as predictors of survival in advanced stages. The *TGF*-β signaling pathway plays a vital role in the control of proliferation, differentiation, apoptosis, and invasion of tumor cells. Of the 140 miRNAs known to regulate genes in this pathway, those associated with two years' overall survival were used to create a panel comprising 17 miRNAs in order to derive a score for

Table 2 Selected circulating miRNAs associated with prognosis of NSCLC patients

miRNA	Expression level of target miRNA	Effect observed	Material	References
let-7	Down-regulation	Poor prognosis overall survival	Plasma	Yu et al. (2008)
miR-16	Overexpression	Good prognosis overall survival	Plasma	Wang et al. (2013)
miR-17-5	Overexpression	Poor prognosis overall survival	Plasma	Chen et al. (2013)
miR-19	Overexpression	Poor prognosis overall survival	Plasma	Lin et al. (2013)
miR-21	Overexpression	Poor prognosis overall survival	Plasma	Robles et al. (2015)
miR-137	Overexpression	Poor prognosis overall survival	Plasma	Yu et al. (2008)
miR-126	Down-regulation	Higher risk for progression	Plasma	Sanfiorenzo et al. (2013)
miR-155	Overexpression	Higher risk for progression	Plasma	Sanfiorenzo et al. (2013)
miR-486	Down-regulation	Higher risk for relapse	Plasma	Li et al. (2015)

patients with a high risk of death. Notably, the abundance of *miR-16* was shown to be associated with a significantly longer overall survival of the patients (Wang et al. 2013).

MiR-21 can, in combination with the expression of four protein-coding genes (*YPO1*, *BRCA1*, *HIF1A*, and *DLC1*) as well as *HOXA9* promoter methylation, be used to divide patients with lung carcinoma into two groups differing by their survival rates, even with stage I tumors (Robles et al. 2015). While every single one of the aforementioned factors is prognostic (Hazard ratio (HR) between 2.3 and 3.0), the combination provides a much more accurate result (HR 10.5) (Robles et al. 2015).

The *let-7* family was the first miRNA family to be identified in humans (Pasquinelli et al. 2000). In lung cancer, it has been shown that *let-7* inhibits the expression of oncogenes such as *RAS*, *MYC,* and *HMGA2*, that are known to be important for cell proliferation (Pasquinelli et al. 2000; Johnson et al. 2007). *Let-7* also inhibits the expression of the cell cycle control gene *CDK6*. Thus, reduced expression of *let-7* leads to a promotion of cell cycle progression. Furthermore, *let-7* demonstrated to regulate the *DICER1* gene expression (Johnson et al. 2007), which is vital for miRNA biogenesis, indicating that *let-7* might function as a general regulator for miRNA biogenesis (Tokumaru et al. 2008).

3.3 Predictive Circulating MicroRNAs in Lung Cancer

Surgical resection of early diagnosed, localized tumors is by far the most promising treatment option for NSCLC patients as it is correlated with substantially improved prognosis (Padda et al. 2014). Nevertheless, post-surgical recurrence and metastases rates are high (30–70% depending on the tumor stage (Martini et al. 1995, 2002). Accordingly, intensive follow-up sampling is crucial to detect recurrence after surgery and to reduce mortality (Westeel et al. 2000). In recent years, considerable efforts were made to implement circulating miRNAs as biomarkers for post-operative tumor monitoring in NSCLC patients (Table 3). In most of these studies, post-surgical recurrence was monitored under the assumption that tumor-associated circulating miRNAs decrease after surgical removal of the tumor: Le et al. showed for the first time, that the abundances of *miR-21* and *miR-24* were lower in post-operative compared to pre-operative samples but not different from normal controls. This suggests their potential utility as biomarkers for recurrence after surgery (Le et al. 2012). Similar results were obtained for a miRNA panel consisting of *miR-205*, *miR-19b*, *miR-30b*, and *miR-20a* (Aushev et al. 2013) as well as for *miR-486*, whose down-regulation after surgical tumor resection was associated with long recurrence-free survival (Li et al. 2015). In 2015, the so far most comprehensive longitudinal analysis of circulating miRNAs in post-operative NSCLC patients was conducted (Leidinger et al. 2015). A total of 1205 miRNAs in 26 lung cancer patients were monitored over a period of 18 months with up to eight sampling time points per patient. Here, the total number of detectable circulating miRNAs (miRNome) of the post-surgical sampling time points was compared to the pre-surgical sample. The miRNome of patients who later developed metastases was less affected by surgery than the miRNome of non-metastatic patients. These data indicated that lung cancer could globally and stably affect the miRNome of a patient, which is altered upon successful therapy (Leidinger et al. 2015).

In localized lung tumors, high-dose radiation therapy has proven to be a highly effective treatment option, showing improved overall and recurrence-free survival (Chang et al. 2015). However, in NSCLC patients with advanced tumors, radiation therapy has been less successful. In those cases, the outcomes are still poor, and the three-year overall survival ranges between 5 and 20% (Aupérin et al. 2010). Thus, biomarkers with the ability to determine the degree of tumor sensitivity to radiotherapy would be highly desirable to achieve higher treatment precision. Radiation dose and site could be selected more accurately and potentially improve the therapy outcome and reduce side effects due to radiation. Many in vitro studies showed that the down- or up-regulation of specific miRNAs results in sensitivity or resistance to ionizing radiation of lung cancer cell lines (Ma et al. 2016; Salim et al. 2012; Tian et al. 2016). Based on these findings, clinical studies aimed to predict radiosensitivity using the abundance of circulating miRNAs in NSCLC patients. One such trial monitored 54 radiotherapy-receiving NSCLC patients over a period of 15 months (Chen et al. 2016) separating the patients into four groups: complete responders, partial responders, stable disease, and progressive disease. The increased abundance of a four-miRNA panel (*miR-98-5p*, *miR-302e*, *miR-495-3p*,

Table 3 Selected predictive circulating miRNAs in NSCLC patients post-treatment

miRNA	Treatment	Expression level of target miRNA	Effect observed	Starting material	References
miR-21	Surgery	Overexpression	Decreased abundance in patients with prolonged recurrence-free survival	Serum	Le et al. (2012)
miR-205	Surgery	Overexpression	Decreased abundance in patients with prolonged recurrence-free survival	Serum	Aushev et al. (2013)
miR-302e	Radiotherapy	Overexpression	Higher abundance in radiosensitive NSCLC patients	Plasma	Sun et al. (2017)
miR-200b	Radiotherapy	Overexpression	Higher abundance in radiosensitive NSCLC patients	Serum	Chen et al. (2016)
miR-210	Cisplatin	Overexpression	Lower abundance in chemotherapy responders	Serum	Li et al. (2013)
miR-21	Cisplatin	Overexpression	Lower abundance in chemotherapy responders	Plasma	Gao et al. (2012)
miR-21	*EGFR*-specific TKI therapy	Overexpression	Higher abundance in TKI resistant patients	Serum	Li et al. (2014)
miR-122	*EGFR*-specific TKI therapy	Overexpression	Lower abundance in *EGFR* mutated patients (compared to wild-type *EGFR*)	Plasma	Zhang et al. (2013)

and *miR-613*) circulating in the plasma of these patients could be applied to separate complete and partial responders from patients with stable or progressive disease (Chen et al. 2016). Sun et al. extended these approaches by combining an eleven-miRNA panel (including *miR-205*, *miR-22*, and *miR-125b*) with clinical factors (ionization dose, adjuvant chemotherapy, age, stage, and Karnofsky performance status) to predict radiosensitivity in NSCLC patients. The combination of the miRNA panel and clinical factors was then successfully used to separate responders from non-responders (Sun et al. 2017). Thus, these studies demonstrated promising results for the application of circulating miRNAs as part of a multi-marker model for the prediction of response of NSCLC patients to radiotherapy.

For localized tumors, systemic chemotherapy is often used as an adjuvant with curative intent after surgery or radiotherapy. In case no targetable driver mutations are present, advanced-stage patients receive chemotherapy as first-line treatment, which is associated with prolonged overall survival and improvements in disease-related symptoms (Pöttgen et al. 2007; Sculier and Moro-Sibilot 2009). The ability of cancer cells to become resistant to chemotherapeutic agents is a considerable impediment for treatment success and a major reason for varying response rates among patients. In vitro, multiple miRNAs are reportedly associated with

chemosensitivity and resistance. Consequently, several studies using circulating miRNA abundances in blood as chemotherapy response predictors have been conducted. For example, circulating *miR-21* (Gao et al. 2012), *miR-210* (Li et al. 2013), and *miR-125b* (Cui et al. 2012) were shown to be significantly more abundant in the groups, which were non-responsive to cisplatin-based chemotherapy in NSCLC patient cohorts. Similar findings were observed with a four-miRNA panel (*miR-25*, *miR-21*, *miR-27b*, and *miR-326*), which were higher in the blood of patients responding to cisplatin-based and pemetrexed chemotherapy (Zhu et al. 2016a). Accordingly, these miRNAs could be used to screen for chemosensitive patients prior to the therapy start and patients who are likely to be non-responders could be spared an unsuccessful and harmful treatment. However, the mechanisms as to how the above-mentioned miRNAs affect resistance and response have not been fully elucidated. *MiR-21* is the most frequently described miRNA. Its ability to predict chemotherapy response has not only been described in serum and plasma but also in tissue samples of lung cancer patients (Xu et al. 2018). Additionally, a down-regulation of *miR-21* in A549 lung adenocarcinoma cells results in a considerably higher expression of the tumor suppressor *PTEN* and decreased expression of the anti-apoptotic gene *BCL2*. Mutation or loss of *PTEN* is frequently observed in several human malignancies and is known to play an important role in the development of chemotherapy resistance (Gao et al. 2012). Despite these encouraging findings, an individual biomarker alone is unlikely to be sufficient to predict chemotherapy resistance, and multi-marker panels might provide more accurate predictions.

In contrast to chemotherapy, tyrosine kinase inhibitors (TKIs) are directed against specific (mutated) molecular targets exclusively found in malignant cells. Therefore, these drugs lead to selective cancer cell toxicity, while sparing normal tissue (Strebhardt and Ullrich 2008). Newly diagnosed patients with advanced lung adenocarcinoma are routinely tested for *EGFR* mutations, *ALK* fusions, and, more recently, also for alterations in genes such as *ROS1*, *RET*, *MET*, *BRAF,* and *HER2*. Targeted therapeutics for these genetic aberrations are available and used in the clinic. Alterations in circulating miRNA abundances have been used to identify patients with *EGFR* mutations and to monitor disease progression upon treatment. The literature covering the role of miRNAs affecting the other targetable mutations is mainly restricted to in vitro studies. An example for circulating miRNAs as biomarkers for targeted therapy is the reduced *miR-122* abundance, which was successfully used to distinguish *EGFR* mutated from *EGFR* wild-type tumors. Accordingly, *miR-112* has potential for predicting the susceptibility for *EGFR*-specific TKIs (Zhang et al. 2013). Similar results were shown for *miR-21* and *miR-10b*, whose increased abundances correlated with the *EGFR* mutation status. Moreover, patients benefitting from the *EGFR*-specific TKI gefitinib are predicted by reduced *miR-21* abundance (Shen et al. 2013).

A major limitation of TKI treatment in NSCLC is the development of resistance mutations and subsequent drug failure, followed by disease progression. This issue has also been addressed in the context of circulating miRNAs. Wang et al. showed an association between the up-regulation of a three-miRNA panel (*miR-21*,

miR-27a, and *miR-218*) and the development of *EGFR*-TKI resistance (Wang et al. 2015b). For *miR-21*, these findings could be confirmed in an additional study, showing increased *miR-21* abundance in *EGFR*-TKI-resistant patients (Li et al. 2014a). Furthermore, high levels of *miR-200c* were associated with increased benefit from *EGFR*-specific TKIs (Li et al. 2014b). The mechanisms behind the predictive value of these miRNAs are yet to be discovered.

4 Summary and Outlook

The development of novel diagnostic, prognostic, and predictive biomarkers is essential to improve survival rates of NSCLC patients. Liquid biopsy is an appropriate source of cancer-derived material. It is minimal invasive and represents tumor heterogeneity better than single tissue biopsies. miRNAs in liquid biopsies have been shown to reflect pathology-associated concentration changes. Because of their high stability, circulating miRNAs are a promising class of molecules for clinical diagnostics. Although the variety of different sample processing options as well as the missing consensus toward normalization of obtained data has led to discrepancies between studies, recent data from multiple studies support the potential of circulating miRNAs as biomarkers in NSCLC. Abundances of individual and multi-marker panels of circulating miRNAs have been described as diagnostic (e.g., *miR-21*, *miR-126*, and *miR-205*), prognostic (e.g., *miR-21*, *miR-16*, and *let-7*), and predictive (e.g., *miR-21*, *miR-122*, and *miR-205*) biomarkers. Still, due to their pleiotropic character and the lack of specificity with respect to tissue types or malignancies, the potential validity of individual miRNAs is a challenge for clinical applications. In an experimental setting it is possible to use single miRNAs to distinguish malignant tissues: For example, several studies have shown that an overexpression of *miR-205* in tumor tissue is able to discern between adenocarcinoma and squamous cell carcinoma with high specificity. However, this is not possible in body fluids, although a higher abundance is indicative for the presence of NSCLC. Thus, the exact mechanisms and changes in cellular metabolism that are caused by overexpression or repression of miRNAs are not fully understood. This is due to the fact that the effects of very few miRNAs have been functionally characterized in sufficient depth so far. Thus, in order to translate circulating miRNAs and miRNA panels into the clinics, there is an urgent need to extend basic research toward functional analysis of miRNAs. Knowing the targets of an individual miRNA is key to evaluate its specificity for affecting the expression of tumor-related genes. In a similar way, the information provided by potentially diagnostic miRNA signatures might facilitate the understanding of specific molecular processes in malignant tissues.

While miRNA panels have demonstrated enhanced sensitivity and specificity when compared to the analysis of individual miRNAs, they can also be combined with imaging markers, e.g., low-dose computer tomography (LDCT). Furthermore, miRNAs could provide important markers adding to the quantification of

cancer-specific driver mutations in ctDNA, a possibility which is currently being investigated in studies aiming at lung cancer diagnosis and treatment monitoring. The potential of marker panels including further information from proteins, lipids, or metabolites, remains yet to be elucidated. In summary, with detailed understanding of miRNA function and by combination with other biomarkers, circulating miRNAs may become important to guide clinical decisions in the future and will contribute considerably to improved diagnosis and survival of lung cancer patients.

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Extracellular Vesicles: Recent Developments in Technology and Perspectives for Cancer Liquid Biopsy

Irina Nazarenko

Abbreviations

EV Extracellular vesicles
ISEV International Society of Extracellular Vesicles

1 Introduction

Genetic and epigenetic changes occurring in tumor cells during the disease progression severely influence the profile of the secreted factors. Consequently, analysis of the cell secretome at least partly may provide information about the changes occurring in the cell of origin. Considerable achievements of the last decades demonstrated that factors secreted by the tumor cells into the circulation might serve as highly potent biomarkers. Although the whole complexity of secreted entities is not completely deciphered, roughly, they can be divided into two large heterogeneous categories, namely molecules directly secreted into the extracellular space and components which are released in a protected form, enclosed into the extracellular membrane vesicles. While the non-vesicular components of the secretome, e.g., hormones and cytokines (Hammes 2003), free-circulating nucleic

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acids including DNA and miRNA complexes with HDL and Ago (Mahn et al. 2011; Vickers et al. 2011) and extracellular nucleases (Deindl et al. 2009) are discussed in other chapters, in this chapter, we focus on extracellular vesicles (EVs).

Thus, EVs are represented by several types of organelle-like structures containing proteins, lipids, nucleic acid species encapsulated in a lipid bilayer originated either from the intracellular membranes of the endosomal compartment or directly from the outer cell membrane (Fig. 1) (Yanez-Mo et al. 2015; Liu et al. 2019; Nieuwland and Sturk 2010). These structures, once emitted from the cell surface, form a heterogeneous population of vesicles, which act as mediators of intercellular communication, transferring a cargo of bioactive molecules from the donor to the recipient cells. Based on the recent achievements, the secreted EVs can be divided according to their origin and content into main categories: exosomes, microvesicles, also referred to as microvesicles/ectosomes (Colombo et al. 2014; Lee et al. 2011), and large oncosomes (Meehan et al. 2016; Minciacchi et al. 2015a, b; Di Vizio et al. 2012; Thery et al. 2018; Rak 2010). Potentially, other types of secreted vesicles exist (Marzesco et al. 2005; Goler-Baron et al. 2012); however, their origin, functions, and content are not clearly defined and therefore will not be discussed in this chapter. Additionally to the secreted vesicles, apoptotic bodies are considered as a type of EVs present in higher amounts in body fluids and therefore

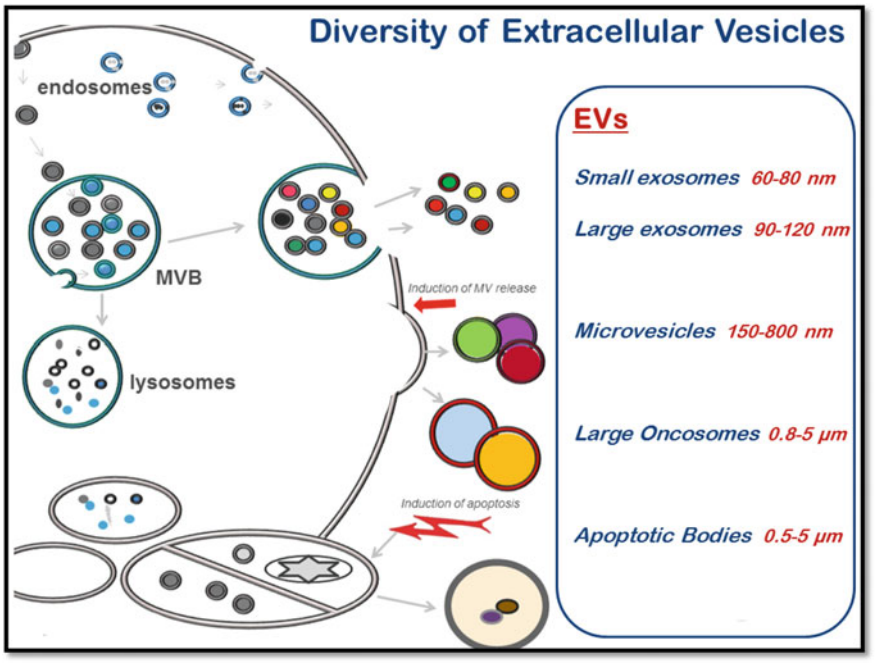

Fig. 1 Schematic presentation of diverse populations of extracellular vesicles (EVs) differed in size and origin

significant for the application of vesicular cargo for diagnostics and prognostics (Grant et al. 2019; Tricarico et al. 2017).

A PubMed search performed in April 2019 shows a steadily growing interest to EVs as a source for diagnostic biomarkers, as the number of publications is increasing from 122 in 2011, the year of ISEV foundation, to 976 publications in 2017. To note is the drop in a number published work in 2018 to 736 in total if all keywords: exosomes, microvesicles, and extracellular vesicle are calculated together (Fig. 2). Technical difficulties and elaboration of current efforts on the work with EVs for their purification and characterization may account to that drop, underlining the need of technological know-how, discussed later on in this chapter.

Specifically, as a liquid biopsy source, EVs were considered from 2014 with 6 pioneer publications, while four years later in 2018, 97 experimental works were published on EVs to this topic (Fig. 3). However, liquid biopsy approaches addressing distinct EV types are still hampered by the lack of EV-type specific isolation methods (Thery et al. 2018). Thus, majority of the studies, carried out so far, worked either with crude EV fractions containing a high portion of non-EV, mostly lipoprotein impurities recovered for instance by precipitation-based methods

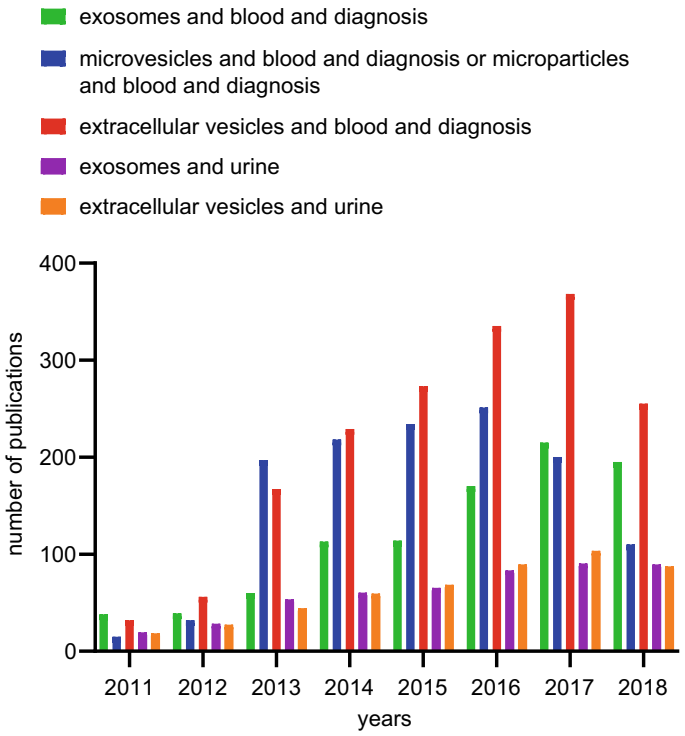

Fig. 2 Publication frequencies of studies investigating EVs as a biomarker carrier for cancer diagnosis according to the PubMed search, April 2019

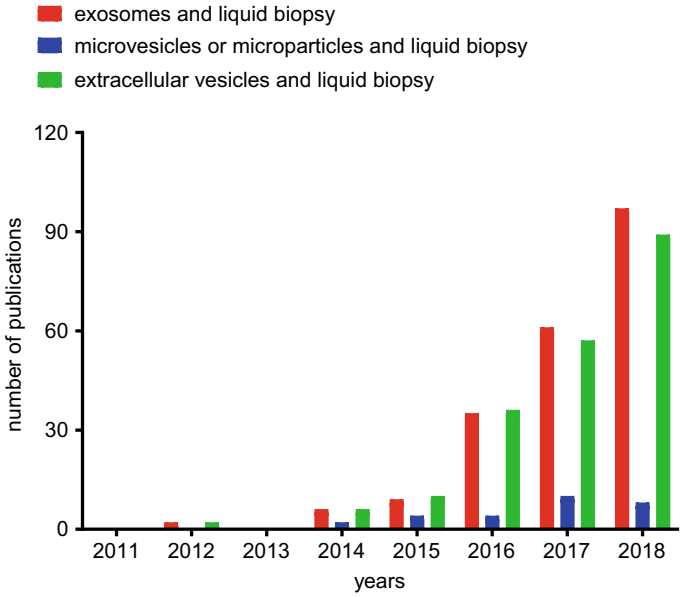

Fig. 3 Publication frequencies of studies investigating EVs in the context of liquid biopsy according to the PubMed search, April 2019

and ultracentrifugation, or EV fractions purified at least partly from lipid contaminants, e.g., by the size exclusion chromatography or density gradient (Simonsen 2017; Johnsen et al. 2018). Furthermore, yet not sufficiently assessed co-purification of EVs and virus particles from body fluids is possible and may lead to false-positive EV biomarkers of viral origin (Ramirez et al. 2018; Raab-Traub and Dittmer 2017). To overcome these limitations, the ISEV community envisions new technologies allowing miniaturization, high-purity EV isolation, and detection. Emerging laboratory-on-chip microfluidic devices (Liga et al. 2015; Wunsch et al. 2016; Kanwar et al. 2014), novel nanomaterials with designed properties (Kabe 2019; Im et al. 2015) and new more sensitive detection methods (16) were already successfully introduced in EV field (Ramirez et al. 2018; Li 2018; Shao et al. 2018). These methods may substantially foster the establishment of EV-based liquid biopsy diagnostics in the clinics and will be discussed below.

2 Emerging Diversity of Extracellular Vesicles

2.1 Exosomes

Exosomes are a population of small nanosized EVs, origin from a portion of multivesicular late endosomes, referred to as multivesicular bodies (MVBs)

(Piper and Katzmann 2007). Secretion of the exosomes occurs upon the fusion of MVBs with the cell membrane (Yanez-Mo et al. 2015; Raposo and Stoorvogel 2013). It is likely; several pathways are involved in the formation of intraluminal vesicles in the late endosomes, among them endosomal sorting complex required for transport (ESCRT) machinery (Babst 2011), which may associate with the syndecan–syntenin–ALIX axis (Baietti et al. 2012), a tetraspanin-dependent pathway (van Niel et al. 2011), and the ceramide/nSMase2-regulated pathway (Trajkovic et al. 2008; Tang 2017; Phuyal et al. 2014). The subsequent event, the fusion of MVBs with the cell membrane leading to the exosome secretion, is likely to be controlled by the RAB family of small GTPases; among them, Rab27 (Ostrowski 2010), Rab11 (Pavarotti et al. 2012), and Rab35 (Hsu et al. 2010) were functionally characterized. Additionally, SNARE proteins are involved, as reviewed by Bobrie et al. (2011). Due to their origin, exosomes exhibit enrichment in particular proteins, e.g., tetraspanins CD63, CD9, CD81, major histocompatibility complex I (MHCI), tumor susceptibility gene 101 (TSG101), and syntenin-1 (Kowal et al. 2016). It is likely that also recruitment of nucleic acid is tightly regulated and specific motifs may enhance recruitment of microRNAs to the exosomes (Villarroya-Beltri et al. 2013). This selectivity of exosome cargo leads to differences between exosome and donor cell content (Liga et al. 2015; Wunsch et al. 2016; Kanwar et al. 2014; Kabe 2019; Im et al. 2015; Li 2018; Shao et al. 2018). However, a characteristic pattern of proteins and nucleic acids of a donor cell is well detectable as a "fingerprint" or a "signature" in the exosomes (Yanez-Mo et al. 2015; Simons and Raposo 2009; Nazarenko et al. 2010; Nawaz et al. 2014), which is particularly crucial, given the application of exosomes for liquid biopsy.

2.2 Microvesicles

Microvesicles are a designation frequently used for a wide range of different types of extracellular vesicles. In this chapter, we adopt the definition of microvesicles as a heterogeneous population of vesicles of 100- to about 600-nm diameter which, in contrast to exosomes, are released by direct budding from the cell surface upon different stimuli (Yanez-Mo et al. 2015). Microvesicles, released by the stimulated platelets or endothelial cells and endowed with pro-coagulant and pro-inflammatory properties, are frequently designated as microparticle (Ridger et al. 2017). Several publications describe a heterogeneous population of tumor-derived microvesicles of 100–400-nm diameter released from the tumor cells, and containing oncogenes was defined as oncosomes (Meehan et al. 2016; Rak and Guha 2012).

Different stimuli such as shear forces, changes in Ca level, and hypoxia can regulate the release of microvesicles (Allan et al. 1976; Shukla et al. 1978). There are first pieces of evidence, suggesting that also the content of microvesicles may at least partly be regulated in a stimuli-dependent manner; e.g., enrichment of the antigen LAIR-1 on microparticles produced by monocytic leukemia cells was observed exclusively upon stimulation of the cells with P-selectin chimera but not

with LPS (Bernimoulin et al. 2009). It is likely that the asymmetric lipid distribution on the cell surface and the mitochondria-dependent stimuli play a role in the production of microvesicles. Thus, gelsolin, translocase, floppase, scramblase, and calpain were shown to be involved in microvesicle release (Alexandru et al. 2017). Similar to the processes during the apoptosis induction, an increase of intracellular calcium leads to a mitochondria-mediated caspase-driven cytoskeleton degradation, inhibition of flippase and activation of floppase, allowing a "flip-flop" of the membrane phosphatidylserine (PS) residues (Connor et al. 1992). These events are followed by the shedding of microvesicles, decorated with PS residues on their outer surfaces (Chung et al. 2007). Also, the number of shed microparticles may have a diagnostic value; thus, the Scott syndrome, appearing as a bleeding disorder, is likely being a defect in the lipid scramblase in the hematopoietic stem cells and causes a deficiency of PS exposure on platelets, accompanied by the impairment of microparticle shedding (Morel et al. 2010).

2.3 Large Oncosomes

Additionally to the extracellular vesicles of nano- and submicron size described above, large vesicles over 1-μm diameter, which are released from the ameboid-like tumor cells and contain metalloproteinases, RNA, ARF6, were described in several cancer types, e.g., prostate cancer, bladder, and glioblastoma (Meehan et al. 2016; Di Vizio et al. 2012). They are likely to contain distinct protein and miRNA cargo, are different from smaller vesicles, and represent a separate EV population (Minciacchi et al. 2015). These vesicles were designated as large oncosomes. However, not many data are available about this subtype of EVs.

2.4 Apoptotic Bodies

Apoptotic bodies in contrast to the exosomes and microvesicles are not secreted by the cells but formed in response to the apoptotic signals. This irreversible cascade starts with the caspase-driven cytoskeleton cleavage, chromatin condensation, followed by the cell shrinkage, membrane blebbing, DNA fragmentation, and finally vesiculation and formation of the apoptotic bodies (Coleman et al. 2001). The latter are membrane particles of approximately 0.5–3-μm diameter, containing random fragments of the cell cytoplasm and fragmented DNA (Hashimoto et al. 1998; Ihara et al. 1998). Once being released, apoptotic bodies are phagocytosed by the adjacent cells or by the macrophages. The increasing evidence supports the transfer of bioactive molecules from the apoptotic to the recipient cells, e.g., during age-related cartilage calcification (Hashimoto et al. 1998) or in the mediation of immune suppression in cancer (Xie et al. 2009). There is evidence that the DNA from apoptotic bodies can be transferred into the nuclei of recipient cells (Holmgren 2010). Other nucleic acid species, such as mRNA and miRNA, maintain their functionality and are detectable in the cytoplasm (Zernecke et al. 2009). Altogether,

the cargo of bioactive molecules enclosed in apoptotic bodies can contribute to an adequate response of the organism on the apoptotic stimuli. In cancer, the large number of apoptotic bodies is daily shed into the circulatory system (Sleeman et al. 2011). Furthermore, chemo- and radiotherapy-induced apoptosis increase a proportion of apoptotic bodies in the blood of cancer patients considerably enhancing their relevance in the mediation of systemic effects during disease progression and therapy and consequently supporting their relevance for liquid biopsy.

3 Detection of Specific EV Cargo in the Context of Liquid Biopsy Studies

New insights into EV biology and functions underpinned their high potential to offer new diagnostics options. Cells shed various EVs supplemented with a specific profile of bioactive molecules protected from the extracellular proteases, shear stress, and other external forces. They are determined for the regulation of distant recipient cells and consequently contain representative information about the cell of origin. The EVs are distributed among body fluids, including blood, urine, saliva, cerebrospinal fluid, breast milk, ascites, and other, composing thereby an easily available source of biomarkers for health and disease state. Here, we provide an overview of different EV components identified as potential biomarkers for cancer.

3.1 Protein Detection

Proteins are one of the best-investigated EV components. Among them, membrane proteins, chaperones, components of cytoskeleton, growth factors and their receptors, metabolic enzymes were detected.

3.1.1 EV Protein Cargo

On the one hand, the release of signaling molecules within the EVs allows for their specific delivery to the recipient cells. This mechanism is likely being used by tumor cells, which produce different types of EVs for initiation and support of local and systemic effects as modulation of tumor stroma and formation of the pre-metastatic niches (Peinado et al. 2012), organization of tumor vasculature, e.g., by the release of Dll4 (Sheldon et al. 2010) and activation of endothelial-like progenitors (Nazarenko et al. 2010), mediation of immune suppression (Mignot et al. 2006), and deregulation of blood coagulation (Rak 2010). On the other hand, secretion of specific proteins within the extracellular vesicles serves as a powerful mechanism for the modulation of the activity of entire intracellular signaling pathways in the donor cells by direct sequestration of its regulators into EVs. Thus, the Wnt pathway can be antagonized in the donor cell by the release of β-catenin within the EVs (Chairoungdua et al. 2010).

Consequently, the EV protein cargo delivers at least two types of information: firstly, the set of proteins specifically sorted to EVs in order to mediate changes in recipient cells and causing changes on systemic levels, and secondly, the set of proteins, which were actively removed from the cell to EVs in order to switch individual signaling cascades, thus reflecting the entire cellular processes in the donor cells. Improvement of our knowledge about the EV protein cargo and creation of intelligent databases, allowing protein functional annotation, may provide beneficial information in terms of liquid biopsy and predictive biomarkers for systemic changes and intra-tumor features, as molecular signatures of primary tumor and metastases reflecting their heterogeneity and consequently providing useful information about reply to particular type of therapy.

3.1.2 Current Knowledge of EV Proteins in Biofluids

The available literature on protein EV content was extensively reviewed in several recent works (Rak 2013; Zhang et al. 2018a; Belov et al. 2016), and the majority of studies are implemented in the EV databases ExoCarta (Liang et al. 2013) and EVpedia (Kim et al. 2015). However, it should be noticed that the data available so far were generated based on the analysis of EVs isolated from conventional cell cultures. Indeed, there is first evidence demonstrating differences between EVs released under 2D and 3D conditions (Rocha et al. 2019), indicating consequently that protein content of EVs released by in vivo tumor may differ from the protein content of EVs produced by the corresponding tumor cells, growing in the 2D cell culture. Analysis of pure tumor EVs isolated from blood or other biofluids is highly beneficial; however, due to the methodological challenges, it is hampered by the vast amount of other components, mainly lipoproteins and soluble protein complexes, which are co-isolated together with EVs due to the similarity in size, density, and charge (Thery et al. 2018; Simonsen 2017; Johnsen et al. 2018). Consequently, it cannot be excluded that proteomics data available so far may contain a particular portion of proteins, co-isolated with EVs as impurities. Further development of the isolation and separation techniques will be required for a comprehensive characterization of pure EV populations isolated from the biofluids.

3.1.3 Potential to Implement EV Protein Analysis in Liquid Biopsy

Taking into account that nearly every cell of the body releases EVs into the extracellular space, EVs of different sources comprise body fluid—EV landscape. These EVs harbor a corresponding surface signature of the cell of origin (Fais et al. 2016). Establishment of these signatures for each particular EV source may substantially foster the implementation of EV analysis in liquid biopsy. Several candidate surface biomarkers were successfully tested in in vitro and pilot clinical settings. Thus, CD47, CD71, and EpCAM were detected on ovarian cancer-derived EVs (Zaborowski 2019); additionally, EpCAM and CD147 were detected on the colorectal cancer-derived EVs (Tian et al. 2018); while EpCAM was cleaved from the surface of the EVs derived from the breast cancer (Rupp et al. 2011); PSMA was found to be increased in EVs in patients with prostate cancer (Park et al. 2016);

EVs derived from the blood samples of CLL (chronic lymphocytic leukemia) contain increased levels of CD5, CD19, HLA-A, B, C (44). Furthermore, (Belov et al. 2016) glypican-1, located on EVs, was characterized as an ultimate biomarker for pancreatic cancer (Melo et al. 2015); in an alternative study, CKAP4 was suggested as an EV biomarker for pancreatic cancer (Kimura et al. 2019). However, none of the characterized biomarkers is established and approved for clinical application yet. Development of methods allowing a direct EV characterization from a biofluid sample, e.g., nanoflow cytometry or laboratory-on-chip devices, may allow the introduction of EV analysis into clinical routine. Some of the methods will be discussed in the next part of this chapter.

3.1.4 Detection of Soluble Protein Ligands in EVs

Interestingly, ligands initially thought being released exclusively as soluble proteins can also be transported within the secreted vesicles. Thus, amphiregulin, heparin-binding EGF-like growth factor, epidermal growth factor, and transforming growth factors were detected in breast cancer EVs, suggesting their role in the formation of the pre-metastatic niche (Peinado et al. 2012). S100A4 can be released as a soluble factor and within EVs, promoting tumor progression (Forst et al. 2010). Similarly, in pancreatic cancer cells, while the 110 kDa-truncated EGFR is secreted as a soluble protein, the 170 kDa full-length protein, 65 kDa intracellular kinase domain was detected in EVs (Adamczyk et al. 2011), underlining the significance of both, the free-circulating fraction and the EVs for the application in liquid biopsy. One of the most visible EV components relevant to their diagnostic value is tissue factor (TF) showed in many studies being not only released as a soluble protein but also harbored by EVs and associated with the regulation of clotting system and coagulant properties of different body fluids (Atkinson et al. 2013; Beer et al. 2016; Bernard 2019; Elsherbini and Bieberich 2018; Bastida et al. 1984). Furthermore, EVs are likely to serve as a transport platform for proteases and their substrates. The L1 adhesion molecules are cleaved by ADAMs within the EVs in the ascites of patients with ovarian carcinomas and then released as soluble proteins (Keller et al. 2009; Stoeck et al. 2006).

3.2 Detection of Long and Short RNAs in EVs

Ceccarini et al. delivered preliminary pieces of evidence of a horizontal transfer of mRNA via EVs, showing “particulation” of extracellular mRNA released by the colorectal carcinoma cells (Ceccarini et al. 1989). The biological meaning of this phenomenon remained for a long time unclear. Development of better detection and purification techniques allowed isolation and analysis of EV RNA (Duijvesz et al. 2011) and led to the detection of biomarkers associated with cancer (Alderton 2012; Ciardiello et al. 2016; Cocucci et al. 2009; D’Asti et al. 2016; Keller et al. 2006), immune response (Bobrie et al. 2011; Liu et al. 2015; Pulliam and Gupta 2015; Whiteside 2016), autoimmune diseases, and fetus sex (Keller et al. 2011). Nowadays, at least two databases, ExoCarta (Cheung et al. 2016) and EVpedia

(Kim et al. 2015), contain comprehensive information about studies on EV RNA. Additionally, EVmiRNA, a database of miRNA profiling in EVs, was recently created and published (Liu et al. 2019).

3.2.1 Coding and Noncoding Long RNAs

Several features are substantiating high relevance of the long RNA EV cargo.

First is enrichment in mRNAs representing *bona fide* donor cells in the EVs (Batagov and Kurochkin 2013; Li 2019; Nazarenko et al. 2013; Ratajczak et al. 2006; Baj-Krzyworzeka et al. 2006). The pattern of these genes is comprised of entire regulators of signal transduction cascades, e.g., membrane kinase receptors and transcription factors. For instance, EVs derived from the embryonic stem cells are up to 10000-fold enriched in mRNAs encoding pluripotency transcription factors Oct4, Nanog, Rex-1, and SCL. Uptake of these vesicles by hematopoietic progenitors leads to the translation of these mRNAs followed by the acquisition of stem cell-like phenotype (Ratajczak et al. 2006). Glioblastoma cells expressing constitutively active oncogene *EGFRvIII* produce EVs containing *EGFRvIII* mRNA. Uptake of these vesicles by the *EGFRvIII*–negative tumor cells leads to the translation of the vesicular *EGFRvIII* mRNA and protein production, resulting in a significant increase of cell tumorigenicity (Skog et al. 2008). Human gastric tumors positive for HER-2/new and MAGE-1 produce EVs enriched in the corresponding mRNAs (Baran et al. 2010).

The second important feature of the EV RNA cargo is a pattern of mRNAs present in the donor cells in low amounts but highly enriched in EVs. For instance, pancreatic EVs with pro-angiogenic properties contain high amounts of *FGFBP1*, *GDF3,* and *CCR7* mRNAs (Nazarenko et al. 2010), which are nearly undetectable in the donor cells (Zhao et al. 2011; Abuharbeid et al. 2006). In this context, enrichment of two mRNAs, KRTAP5-4 and MAGEA3, in blood EVs was reported as diagnostic biomarkers for the detection of colorectal cancer (Dong et al. 2016).

Third important observation refers to the fact that the majority of the works reported so far contain microarray data or PCR analysis, not delivering the information about the length of available transcripts. However, the analysis of RNA fragmentation demonstrates a strong enrichment in 3' untranslated regions (Batagov and Kurochkin 2013), indicating a low number of full-length transcripts and a high number of truncated sequences in EVs in breast cancer (Batagov and Kurochkin 2013; Jenjaroenpun et al. 2013) and glioma primary tumor cells (Wei et al. 2017). Recent data generated in hepatocellular carcinoma supported this, demonstrating that blood EVs contain a low amount of intact mRNAs and high amounts of spliced junctions (Li 2019), suggesting that RNA content may provide unique information about genetic aberrations and tumor-specific splice products. Supporting this, EV enrichment in prostate cancer-specific splice variants of the *AGR2* mRNA was demonstrated in urine EVs of the prostate cancer patients as a highly potent new noninvasive biomarker (Neeb et al. 2014); furthermore, a splice variant of the androgen receptor, *AR-V7*, an indicator of castration resistance, was found in the blood and urine EVs of patients with castration-resistant form of prostate cancer.

(Woo et al. 2018; Seitz et al. 2017). A company Exodiagnos Ltd launched a first noninvasive kit ExoDx Prostate IntelliScore recently for the detection of prostate cancer, which is based on the RT-PCR from urine EVs. Two RNAs, noncoding prostate-specific sequence *PCA3* and a splice product *TMPRSS2:ERG,* are detected in this kit, and for normalization SAM pointed domain-containing Ets transcription factor (*SPDEF*) is used (McKiernan et al. 2016).

Among the long RNA, additionally to the high content of oncogenes and their splice variants, the presence of long noncoding RNA (Chen et al. 2016; Xie et al. 2019) and circular RNA (Fanale et al. 2018) was reported in EVs derived from cell culture and the body fluids. These types of long RNAs are suggested to have high diagnostic potential. A long noncoding RNA BCAR4 was recently shown to improve the performance of noninvasive colorectal cancer detection based on MAGEA3 and KRTAP5-4 mRNA levels in blood EVs (Dong et al. 2016). However, besides these first indications, it was not investigated yet in many details and therefore will not be further discussed in this chapter.

3.2.2 Short RNAs

An idea about the sequestration of excessive regulatory noncoding nucleic acids into EVs was proposed some time ago based on data, showing that tumor suppressor miRNAs of the let-7 family are shed within EVs by tumor cells to maintain their oncogenic potential (Ohshima et al. 2010). We have recently provided data, supporting a hypothesis that tumor cells may use miRNA shedding as a regulatory mechanism for the optimal adaptation to the environment. Thus, the change of cell culture conditions from 2D to 3D resulted in the enrichment of EVs in a specific profile of miRNAs regulating proteins of the ARF6 signaling pathway (Rocha et al. 2019). Consequently, EV miRNAs may provide useful information about progressing tumor and response of tumor cells on occurring changes, e.g., during therapy.

The EV miRNAs were excessively characterized in many works and different systems. However, experimental work demonstrated that method chosen for isolation of EVs and miRNAs has a direct influence on the miRNAs content isolated (Buschmann et al. 2018). In line with this evidence, profile, quality, and quantity of the isolated extracellular RNA from blood and urine were demonstrated to be strongly dependent on the method chosen for the RNA isolation (Srinivasan 2019). Comparative analysis and computation analysis revealed four classes of RNA cargo among different biofluids, including cargo associated with low-density extracellular vesicles, lipoproteins, Ago2 ribonucleoproteins, and vesicular carriers of different densities (Murillo 2019). Disregarding the methodological challenge, nearly for each cancer type multiple studies were performed showing biomarker potential of EV miRNAs as reviewed elsewhere for clear cell renal cell carcinomas (Zhang et al. 2018b), brain tumors (Fontanilles et al. 2018), ovarian cancer (Yokoi et al. 2017), colorectal cancer (Normanno et al. 2018; Fuji et al. 2019), pancreatic cancer (Ko et al. 2018), ovarian cancer (Giannopoulou et al. 2019).

3.3 DNA

3.3.1 Discovery of EV DNA

The first report indicating genomic DNA being present in different EV populations in vitro and in the blood of prostate cancer patients, including mutated oncogenes *P53*, *PTEN*, and *MHL1*, was published in 2014 (Lazaro-Ibanez et al. 2014). Furthermore, the functionality of the transferred DNA was demonstrated on *BCR/ABL* oncogenic DNA transferred by EVs and causing chronic myeloid leukemia in vivo (Cai et al. 2014). Mutated oncogene *RAS* was shown to exhibit an activating effect on DNA shedding within the EVs (Lee et al. 2014).

3.3.2 Application of EV DNA for Liquid Biopsy

Because of the emerging field of liquid biopsy, several studies addressed the usefulness of EV DNA for that purposes. A simple comparison of the amounts of free-circulating DNA versus DNA enclosed in EVs in blood samples revealed that a larger portion of DNA is located in EVs (Fernando et al. 2017; Klump 2017). Furthermore, EVs harboring tumor DNA were demonstrated to cross the blood-brain barrier, thus indicating possible application of EVs for detection of mutated oncogenes in different malignancies, including brain tumors (Garcia-Romero et al. 2017). Supporting this statement, the DNA of mutated *KRAS* and *TP53* was detected in the EVs isolated from the blood of pancreatic cancer patients (Kahlert et al. 2014). There few comparative clinical studies analyzing both free-circulating and EV DNA were performed. These data indicate that in different cancer types, the amounts of DNA of mutated oncogenes enclosed in EVs and those containing in the free-circulating fraction may be different. In pancreatic cancer, both EVs and the free-circulating fraction contain equal amounts of mutated *KRAS* (Allenson et al. 2017), which provides predictive and prognostic values relevant to therapeutic decisions (Bernard 2019).

In contrast to that, in patients with the stage IV melanoma, the free-circulating DNA fraction contained severalfold higher amounts of $BRAF^{V600E}$ DNA as compared to EVs isolated from the same samples of blood, and similar results were showed for distribution of *CKIT* wild-type and mutant DNA by the patients with an aggressive form of mastocytosis (Klump 2017). To explain this phenomenon, it can be suggested, that while the majority of free-circulating DNA is released by the tumor cells undergoing apoptosis, EVs are released from different blood cells and only the minority of them originated from the tumor. EVs contain double-stranded DNA, representing the complete chromosomal set of the human genome (Thakur et al. 2014). Consequently, the portion of mutated oncogenic DNA, corresponding tumor-derived DNA, may be considerably lower as wild-type DNA.

Taking together, vesicular genetic cargo represents a highly attractive source of biomarkers. The challenge of future research will be to characterize molecular mechanisms of delivery of mRNA, miRNA, and DNA into different types of secreted membrane vesicles. Concerning clinical applications, development of robust and reproducible methods allowing substitution of laborious purification steps applied for present studies (Thery et al. 2006) will be required. Fast and

cost-effective approaches allowing sensitive detection of genes and RNA species from low amounts of vesicles or rather directly from the body fluids will facilitate the implementation of Exo and MVs analysis in clinical praxis.

4 Current Challenges in EV Purification from Body Fluids

Despite the high potential of the body fluid EVs for diagnostics illustrated in this chapter, their application in the clinics is not established yet. Here, we exemplify main limitations mostly accounting to technical problems not solved until now. Furthermore, we indicate several emerging technologies, which may improve clinical applicability of blood EVs as biomarkers in the diagnostics.

4.1 Impact of Pre-analytics

While the analysis of cellular blood components counts to well-established techniques, the isolation and purification of the acellular blood fractions, including different types of vesicles, remain still a technical challenge, influenced by many parameters.

Starting with the pre-analytical part, blood draining routine and the choice of a vacutainer-type have a strong impact on the count of EVs due to the ex vivo EV release. A comparative analysis of different vacutainers has shown that usage of acid-citrate-dextrose collection tubes results in a lower ex vivo EV release as compared to other plasma or serum collection tubes (Gyorgy et al. 2014). Furthermore, such parameters as count of remaining after centrifugation platelets and hemolysis should be controlled in the samples, as both may falsify the real EV number and consequently interfere with the quality of downstream analysis (Yuana et al. 2015; Bernet et al. 2011).

It may be a question of future development of better conservation media, allowing the preservation of ex vivo vesiculation, protease and nuclease activities in blood-collecting tubes, which would allow for robust pre-analytical conditions improving reproducibility and reliability of EV biomarker studies.

4.2 EV Purification

By the EV analysis and conclusions made based on the analysis of blood EVs, it should be taken into account that blood is a complex viscous biofluid, containing various components including a high number of lipoproteins, strongly resembling EVs by their biochemical and biophysical properties. Consequently, all currently available one-step methods for EV purification result in a mixture of lipoproteins and EVs, in which the portion of EVs is considerably lower than lipoproteins, and the absolute number of the latest is highly variable and is dependent on parameters

such as age, diet, metabolism, health/disease state (Johnsen et al. 2018). To reduce the portion of lipoproteins and chylomicrons, the blood of fasting individuals may be a better source, which however is not always possible depending on the health condition of a patient. A better purification may be achieved if two-step approaches combining separation by size followed by the separation by density are applied. A combination of iodixanol density gradient centrifugation, allowing to separate EVs from the chylomicrons, VLDL, IDL, and LDL, which have lower density as EVs, followed by the size exclusion chromatography, allowing separation of EVs from the remaining HDL, which have similar density but are considerably smaller, may significantly improve quality of EV purification (Karimi et al. 2018). However, application of two-step purification, such as density gradient centrifugation followed by the size exclusion chromatography is time-consuming and cannot be applied in a routine clinical praxis. Consequently, it cannot be ruled out that potential biomarkers identified so far in patient samples as EV biomarker correspond to components associated with lipoproteins and not with EVs (Johnsen et al. 2018).

Better purification techniques will be required in order to implement the analysis of pure EV populations in clinical samples in large clinical studies. Some developments in nanotechnology, addressing this requirement, will be discussed below.

4.3 EV Quantification

Because the majority of EVs are vesicles of nano- or submicron size, conventional methods for quantitative analysis of biological objects are not applicable. Techniques based on single-particle tracking analysis using light scattering technologies have been manifested in the community for the EV counting according to the worldwide survey 2016 (Gardiner et al. 2016). To these methods belong dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA). The DLS is based on the measurement of bulk scattered light from EVs. Consequently, the result of DLS-provided size distribution is intensity-weighted, and it may tend to overestimate the number of large vesicles present in the solution and to underestimate the real number of small vesicles (Klump 2017). NTA was developed for the calculation of monodisperse nanoparticles in liquids, where the light beam is used for particle illumination and the camera is tracking single-particle motion, which then is subjected to mathematical quantification. Consequently, both NTA and DLS have some limitations in estimating the real number and size of physiological polydisperse mixtures of particles of nano- and submicron sizes (Thery et al. 2018; Johnsen et al. 2018). Current development of the technology is a combination of particle counting with the quantification of EVs labeled with the specific membrane dyes or surface protein biomarkers, which is likely to improve the accuracy in estimation of a real number of small EVs in a sample (Wang et al. 2016; Carnell-Morris et al. 2017).

Tunable resistive pulse sensing (TRPS) was developed as an alternative method for EV quantification. It is based on the measuring of changes in the ionic current generated by the transport of a single vesicle through a tunable nanopore in a

polyurethane membrane. Using nanopores of different sizes, TRPS can be used for a broad range of particles from about 50 nm to 1 μm. However, several measurements of the same sample are required to measure a polydisperse particle mixture. Similar limitations mentioned by NTA would account also for TRPS (Maas et al. 2017; Vogel et al. 2016).

High-resolution flow cytometry was demonstrated as a promising technology for EV quantification and characterization (van der Vlist et al. 2012). While the conventional flow cytometry has limited resolution for particles smaller than 500-nm diameter, the number of small vesicles is underestimated since multiple small EVs are needed to produce fluorescence strong enough to be detected by the cytometer. This phenomenon was described as a swarm effect (Libregts et al. 2018). To overcome this problem, latex or magnetic beads coupled to corresponding antibodies are frequently applied. The method is referred to as bead-assisted flow cytometry and can be used for a semiquantitative EV analysis (Suarez et al. 2017) in the laboratories equipped with a conventional flow cytometer.

Considerable efforts are undertaken in the ISEV scientific community to standardize the application of flow cytometry for EV measurements (de Rond et al. 2018; van der Pol et al. 2018). It was shown that only a limited number of instruments available on market instruments can measure EVs below 400 nm in diameter. Furthermore, the impact of EV preparations and instrument characteristics have a high impact on the outcome of the measurements and changes of these parameters may lead to different results produced on the same samples (Wiklander et al. 2018).

To address the challenge of measuring of nano-objects by flow cytometry, high-resolution flow cytometries were developed and tested in pilot clinical studies, showing promising results (Tian et al. 2018; Kabe et al. 2018).

However, a comparative analysis of NTA, TRPS, and flow cytometer shows differences in the quantification of a particle number (Maas et al. 2015) and suggests that for EV characterization, processing of samples and EV purity play a decisive role for the reproducibility of the results. Application of several techniques is advantageous, as none of the current methods provides a complete picture of a true EV count and size distribution. Further development of technology and instruments and the introduction of reliable reference materials are required to enrich robustness of measurements and applicability for the work with clinical samples.

5 Recent Developments in Nanotechnology Toward Miniaturized Direct EV Isolation

5.1 Integrative Laboratory-on-Chip Methods

There were several miniaturized approaches for EV capture from biofluids and biomarker detection recently developed. Different microfluidic and nanostructured platforms were adapted for EV concentration from the biofluids, followed by either

a direct quantification of surface protein biomarkers or a detection of intravesicular RNA or miRNA sequences after the capture. These approaches are based on variable principles, such as filtration (Liu et al. 2017), nanoscale lateral displacement (Wunsch et al. 2016), nanowire capture (Wang et al. 2013), immuno-isolation (He et al. 2014), viscoelastic flow (Liu et al. 2017), acoustics (Lee et al. 2015), alternating current electrokinetics (Ibsen et al. 2017) and flow field flow, or the asymmetric flow field flow fractionation (Zhang et al. 2018a; Kang et al. 2008). The full variety of these approaches are presented in the recent review (Rana et al. 2018). Here, we describe some main principles of the approaches developed so far.

One of the possibilities for miniaturization of the detecting approach is the application of an alternating current electrokinetic, which was recently demonstrated as a microarray chip (ACE chip) successfully applied for the detection of glioma and pancreatic cancer EVs (Ibsen et al. 2017; Lewis et al. 2018). In this approach, plasma or serum was separated from other non-EV particles directly on a microarray chip using dielectrophoresis. EV detection was done using TSG101 and CD63 antibodies, and for the detection of pancreatic cancer, a fluorescently labeled anti-glypican-1 antibody was used. The same principle was used for the analysis of plasma from patients with colorectal cancer. A difference between patients with and without metastases was detected using this chip (Lewis et al. 2018). Another group used the same principle in combination with microfluidic channels in an independent study for a simultaneous pre-concentration and capture of tumor-derived vesicles derived from breast cancer cells (Cheung et al. 2018).

Additionally, to a direct EV surface biomarker quantification, intravesicular molecules can represent an attractive biomarker. Thus, ACE chip was applied to glioma samples for the detection of mutated EGFRvIII mRNA, which was detected using RT-PCR from the EVs captured on chip (Ibsen et al. 2017). A promising alternative to the ACE chip is the application of a nanoscale deterministic lateral displacement (nanoDLD) principle (Smith et al. 2018). Combined on a chip containing 1024 nanoscale arrays, it allowed for a better concentration and yield of EVs from serum and urine as compared to the available benchmark precipitation and filtration methods (Smith et al. 2018).

Additionally to the scientific developments, there is one commercially available instrument called ExoView^{TM} R100 of the company NanoView Biosciences, offering on-chip EV analysis. The instrument was developed based on the principle of single-particle interferometric reflectance imaging sensor (SP-IRIS). It allows multiplexed phenotyping and digital counting of individual EVs captured on a microarray-based solid phase chip (Daaboul et al. 2016). The capture is immunoaffinity-based and allows thereby for simultaneous quantification of EVs harboring different surface proteins from the same sample, offering herewith the first commercial EV chip-based tool.

5.2 Application of Plasmonic-Based Detection

Parallel to fluorescence detection, alternative technologies, such as plasmonic-based EV detection, were developed, allowing for a labeling-free EV detection and showing high potential for substantial enhancement of sensitivity and specificity. The main achievements of this technology are described elsewhere (Rojalin et al. 2019). Here, we explain the general principle and potential of this technique, which mainly includes surface plasmon resonance (SPR), localized SPR, and surface-enhanced Raman spectroscopy (SERS). The first work in this field demonstrates the application of plasmon resonance for EV detection. The authors called the approach "a nanoplasmonic exosome assay (nPLEX)" and tested it successfully on cell lines and ascites of ovarian cancer patients (Im et al. 2014). This assay is comprised of an SPR chip, consisting of the arrays of periodic nanoholes patterned in a metal film. Each array was functionalized with an EV or cancer biomarker, including EpCAM, CD24, CA-125, MUC18, EGFRR, and HER2. By binding of EVs to the array, a spectral shift was registered.

It is likely that next step, development, and improvement of new 3D plasmon structures can be envisioned, allowing for an increased sensing area and a detection range between 1×10^4 and 1×10^{11} particles/mL (Zhu et al. 2018). Additionally to plasmon resonance-based EV detection, conventional SPR was successfully tested in a different setting for EV detection including SPR imaging (Zhu et al. 2014). In this approach, SPR was combined with antibody microarray used to capture EVs. The changes in refractive index were monitored by CCD camera and translated for quantitative EV analysis. A correlation between the metastatic potential of the cells and the amount of EV released was monitored. Being tested on cell lines, the relevance and applicability of this approach for clinical samples are difficult to estimate and should be tested in a separate study (Zhu et al. 2014).

While first approaches were dedicated for the analysis of surface EV molecules, recently, application of a nanoplasmonic system for intravesicular proteins, called intravesicular nanoplasmonic system (iNPS), was successfully demonstrated and applied for the detection drug-dependent EV signature of ovarian cancer cells (Park et al. 2018).

6 Conclusion and Future Perspectives

EVs represent a highly attractive source of biomarkers. However, their application for liquid biopsy in the clinics is hampered mostly because of the lack of validated techniques allowing for their cost- and time-efficient purification from biofluids.

Detection of tumor-derived EVs using their specific surface signature may become an ultimate method for early cancer detection and population screening in the future. Available studies show that blood of a healthy donor does not contain a considerable amount of EVs harboring epithelial biomarkers. Consequently, the presence or increase of EVs decorated with corresponding epitopes, such as

EpCAM, CD147, HER2, PSMA, may serve as an indication for a malignancy. Improvement and miniaturization of tools, capable of detection of a low amount of targets with high background, should be envisioned for that purposes. Prototypes of such tools described above demonstrate their high potential. Furthermore, analysis of EVs in the duration of therapy may provide a unique option for tumor monitoring (Whiteside 2018). Most likely, it is a question of the next step of the development, implementing described principles into final products, which can be introduced in the clinics.

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Bioinformatics

Computational Analysis of DNA and RNA Sequencing Data Obtained from Liquid Biopsies

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1 Introduction

More than 90% of cancer-related deaths are due to the development of an incurable metastatic disease (www.cancer.gov). A major hurdle to the identification of new therapies that prevent or suppress metastases is the heterogeneity of primary and metastatic lesions (Jacoby et al. 2015). Not only are cancers heterogeneous within each cancer type (for instance, breast cancers are clinically divided into HER2-positive, estrogen/progesteron receptor-positive, and triple-negative), but it

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is also becoming increasingly clear that tumor cells from an individual tumor may constitute a large number of tumor subclones with different genetic profiles (McGranahan and Swanton 2017). Intra-tumor heterogeneity has been shown to be highly variable and driven by up to 8000 different coding variants within primary tumors and between primary and metastatic tumor deposits (McGranahan and Swanton 2017; Johnson et al. 2014). Tumor heterogeneity and fitness differences can even be observed among individual cells. Single tumor cells have been shown to be heterogeneous at the genomic, gene expression (Tirosh et al. 2016) and tumor microenvironment level, for instance displaying varying proportions of immune cell infiltrates (Chevrier et al. 2017). Among the hurdles for effective cancer treatment are also difficulties to achieve early detection of some cancer types (Schiffman et al. 2015), the inability to obtain samples in real time from progressing lesions, and the lack of widespread methods that predict drug susceptibility to targeted agents (Steeg 2016).

In this context, the field of oncology is experiencing extraordinary excitement concerning the advent of liquid biopsies and their potential to improve cancer care. Liquid biopsy refers to the analysis of circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) from body fluids (especially blood samples) of cancer patients as well as healthy individuals (Alix-Panabières and Pantel 2016). CTCs are derivatives of primary and metastatic lesions and can be found in the bloodstream of patients as single cells or multicellular clusters (CTC clusters), the latter being associated with a higher propensity to metastasize (Aceto et al. 2014). More generally, CTCs have proven useful for cancer patient stratification of responders versus non-responders before therapy initiation (Carter et al. 2017), for individualized testing of drug susceptibility in the metastatic setting (Yu et al. 2014), as well as for investigating the metastatic process (Aceto et al. 2014; Szczerba et al. 2019; Gkountela et al. 2019). As two sides of the same coin, ctDNA is playing an increasingly important role in monitoring response to treatment (Diehl et al. 2008; Dawson et al. 2013; Khan et al. 2018), in genotyping tumors noninvasively for therapy selection (Chan et al. 2013; Murtaza et al. 2013; Siravegna et al. 2015; Thompson et al. 2016), and in the detection of minimal residual disease postoperatively and of patients at high risk of developing recurrence (Diehl et al. 2008; Tie et al. 2015, 2016). Potentially, it could also serve as a method to screen healthy individuals to detect cancer before clinical manifestation of symptoms (Gormally et al. 2006). While a number of studies on ctDNA and CTCs have already been performed, leading to their clinical application in a limited number of hospitals, cancer centers, and diagnostic centers, much remains to be learned before their widespread use in cancer-related health care. To this end, together with experimental and technological improvements, the analysis of next-generation sequencing (NGS) data obtained from liquid biopsies will play a pivotal role, ultimately aiming to offer accurate diagnostic and prognostic information, as well as important insights into the biology of metastasis formation.

1.1 DNA Sequencing

Cancer is often regarded as a disease of the genome, so it is not surprising that DNA sequencing has provided pivotal information about its features and development (Garraway and Lander 2013). Currently, three main approaches are used to investigate genetic alterations, and they primarily differ in terms of breadth and depth of coverage and area of application.

First, amplicon sequencing provides high-depth information about preselected genomic regions, totaling up to tens of thousands of bases. This method allows to focus on specific hot-spots and cancer-associated genes, and it is particularly useful to investigate low-frequency mutations because of its deeper coverage and higher sensitivity (Forshew et al. 2012). De novo mutation calling with amplicon sequencing allows the identification of single-nucleotide variants (SNVs) and small insertions and deletions (indels) in the captured regions. Structural variants may be identified if their break points are well known and covered by amplicons, while other aberrations are missed.

Second, whole-exome sequencing (WES) targets all coding regions of known genes (1–2% of human genome). Expanded exome capture kits may also include introns, untranslated regions (UTRs), and additional regulatory elements. In general, WES enables screening mutations that affect coding sequences and that are likely to be involved in a specific disease. Compared to amplicon sequencing, WES generally requires higher input and better sample quality. Variability in probe hybridization efficiency often results in uneven coverage, incomplete capture of the targeted regions (usually <90%) (Meienberg et al. 2015), and reference allele bias (Asan et al. 2011). These biases may complicate the detection of copy-number aberrations (Zare et al. 2017). Additionally, differences exist among exome capture kits, possibly making some of them more suited to particular applications than others (Meienberg et al. 2015; Warr et al. 2015).

Third, whole-genome sequencing (WGS) is designed to provide the least biased overview of the entire genome. This approach facilitates comprehensive analyses, including detection of SNVs, indels, and structural variants across both coding and noncoding regions. Compared to WES, WGS offers more uniform read coverage and balanced allele ratios, increasing the sensitivity of variant detection at the cost of increased sequencing and input DNA requirements (Belkadi et al. 2015; Meynert et al. 2014). Repetitive, low-complexity regions of the genome remain challenging to analyze, especially using short-read technologies (36–250 bp) (Treangen and Salzberg 2011). Emerging third-generation sequencing platforms, with read lengths of up to thousands of base pairs, allow the investigation of such regions and further refine copy-number profiles (Garraway and Lander 2013). A variant of WGS is shallow WGS (sWGS), where copy-number profiles alone are obtained from low sequence coverage (<1×).

1.2 RNA Sequencing

The transcriptome provides information about gene expression and RNA-related events, such as alternative splicing and gene fusions (Wang et al. 2009), and it may also inform about SNVs if they are expressed. Sequencing can be performed in a targeted manner, by focusing on selected regions of the transcriptome and subsets of genes, or more comprehensively by considering the whole transcriptome.

Whole-transcriptome sequencing allows for broad gene expression profiling from virtually all cellular systems. This method is also used for detection of new transcripts, alternative splicing events, and other posttranscriptional modifications that can affect gene function. In the context of cancer, RNA analysis has been widely used to detect aberrant expression patterns and fusion genes, and to investigate mechanisms underlying cancer progression and resistance to therapy (Aceto et al. 2014; Lee et al. 2014).

Approaches to transcriptome sequencing can be classified into full length and tag-based depending on the strategy for quantification. While full-length methods attempt to cover the whole transcript, tag-based protocols focus only on the 5′ or 3′ end, reducing the number of reads required to detect and quantify the transcript. Full-length methods are generally used for study designs comprising a moderate number of samples. Tag-based methods have been recently developed for single cells, enabling the screening of thousands of samples (Zheng et al. 2017; Macosko et al. 2015). In addition, tag-based protocols may be used in conjunction with unique molecular identifiers (UMIs). UMIs are random nucleotide barcodes that are ligated to each transcript and allow tracking of single molecules through the amplification process. Reads belonging to the same UMI family are computationally collapsed retaining only sequence variants common to most, if not all, reads in the family. This removes most amplification and sequencing artifacts, as well as PCR duplicates, yielding cleaner sequencing data. Compared to full-length analyses, tag-based methods allow only gene-level quantification and are thus not appropriate for isoform quantification.

1.3 Parallel DNA and RNA Sequencing

Multi-omics approaches enable comprehensive analyses of cellular processes. Traditionally, each omic measurement has been applied to a different section of the same tumor. However, the extent of intra-tumor heterogeneity in bulk samples implies that such samples may be rather different from each other, and it may be challenging to integrate such data. For this reason, recent efforts have focused on the development of parallel measurements in single cells (Bock et al. 2016). For instance, different strategies now enable concurrent sequencing of both DNA and RNA from a single cell (Macaulay et al. 2015, 2017; Dey et al. 2015). These approaches can be particularly useful for dissecting the causal relationships between genetic variation and transcript levels. Similarly, parallel sequencing of bisulfite-converted DNA and RNA provides valuable insight into the interplay

between methylation and gene expression (Angermueller et al. 2016). However, given the multi-step nature of these protocols, high-quality DNA and RNA inputs are required, as well as careful handling of the samples.

1.4 Bioinformatics Challenges

Sequencing of liquid biopsies can be performed at various scales, each of which is tailored to different applications (Tables 1 and 2). For example, ctDNA analysis is primarily limited by the low tumor content of the samples. Sensitive techniques are

Table 1 Summary of DNA sequencing experiments on liquid biopsies and comparison with bulk tumor samples

Main DNA sequencing approaches for liquid biopsies and comparison with bulk tumor samples			
	Bulk	CTCs	ctDNA
AMP	Detection of SNVs, indels, and SVs (if break points are well defined), including low-frequency subclonal mutations	Compared to WES and WGS, higher depth of coverage and sensitivity	Accurate detection of mutations down to AFs ∼ 0.1%. Requires modeling of noise, error suppression
WES	Detection of SNVs and indels down to ∼2% AF in exonic regions, characterization of CNAs. May detect SVs	WGA required. Detection of SNVs and indels. High dropout levels (10–20%)	Requires high enough tumor content (>5% for multiple samples) Characterization of SNVs, indels, and CNAs. May detect SVs
WGS	Characterization of abundant SNVs and indels (∼10% AF) Detection of SVs, accurate detection of CNAs	WGA required. Detection of SNVs, indels, and CNA. Higher dropout levels due to lower depth of coverage	Requires high tumor content (>20%). Characterization of the most abundant SNVs, indels, SVs, CNAs

AF allele fraction; *AMP* targeted amplicon sequencing; *CNA* copy-number aberration; *indel* short insertion or deletion; *SNV* single-nucleotide variant; *SV* structural variant; *WES* whole-exome sequencing; *WGS* whole-genome sequencing; *WGA* whole-genome amplification

Table 2 Summary of RNA sequencing experiments on liquid biopsies and comparison with bulk tumor samples

Main RNA sequencing approaches for liquid biopsies and comparison with bulk tumor samples		
	Bulk	CTCs
Full length	Gene and isoform quantification	WTA required. Gene and isoform quantification. Small–moderate number of cells (typically less than 500). High dropout levels
Tag-based	Gene-level quantification only	WTA required, amplification with UMIs. Gene-level quantification only. Ability to profile thousands of cells. High dropout levels

WTA whole-transcriptome amplification

thus needed to detect tumor signal and distinguish it from technical noise. In the case of CTCs, it may be difficult to isolate a large number of cells, and identification of somatic aberrations is complicated by artifacts arising from the amplification process. These aspects, together with approaches to overcome them, will be discussed in the rest of the chapter.

2 Analysis of Circulating Tumor DNA

As far as the genomic characterization of the tumor is concerned, the analysis of ctDNA is similar to that of bulk tumor samples: In both cases, a multitude of genomes are profiled together. However, several important features distinguish ctDNA. Among these, two are particularly relevant to computational data analysis, namely the low tumor content and the fragmentation pattern of cell-free DNA (cfDNA).

cfDNA consists mainly of wild-type DNA fragments originating primarily from hematopoietic cells (Sun et al. 2015; Snyder et al. 2016). In cancer patients, cfDNA levels are elevated due to additional contributions from tumor cells. The tumor fraction is usually low, especially when compared to regular tumor biopsies, with median allele fractions lower than 10% for advanced cancer patients and lower than 1% in earlier stages (Diehl et al. 2005; Bettegowda et al. 2014). Because of the paucity of tumor DNA in the circulation, sensitive assays and robust analyses are required in order to detect, track, and characterize ctDNA.

cfDNA displays a characteristic fragmentation pattern, which reflects biological processes and can be exploited both experimentally and analytically. cfDNA fragments that originate from non-cancerous cells are approximately 167 nt long, corresponding to the DNA in a chromatosome. Fainter signals can be observed for multiples of this length, whereas fragments shorter than 167 nt display peaks every 10 nt (Snyder et al. 2016; Mouliere et al. 2018). In contrast, tumor-derived fragments are generally shorter (Fig. 1). While the mechanism behind the difference in fragment length distributions is unclear, this divergence offers the possibility to focus on certain length intervals to enhance tumor signal (Mouliere et al. 2018; Underhill et al. 2016). Fragment size analysis may also help distinguish mutations originating in tumor cells from clonal hematopoiesis mutations. In addition to the length of the fragments, there are regularities in the genomic location of the fragments: The alignment coordinates of the fragments are not distributed uniformly, but rather reflect nucleosome occupancy patterns, from which the tissue of origin and tumor gene expression may be inferred (Snyder et al. 2016; Ulz et al. 2016).

In the following, we consider two main goals of sequencing-based ctDNA analysis, namely the quantification of tumor content (ctDNA as a biomarker) and the noninvasive characterization of the tumor genome (ctDNA as a liquid biopsy).

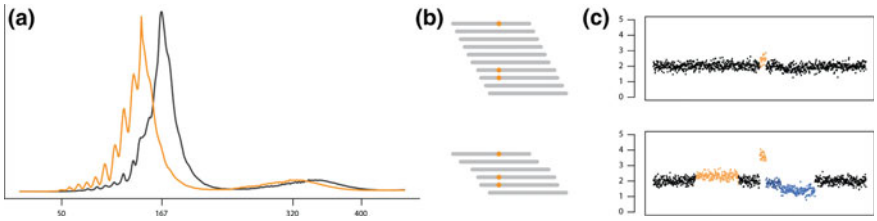

Fig. 1 Overview of sequencing-based ctDNA analysis. **a** Distribution of fragment lengths in cfDNA. Tumor-derived fragments (orange) are generally shorter than molecules from non-cancerous cells (black). **b** Mutation calling. Top: Low tumor content means that somatic mutations (orange dots) occur in a minority of reads (gray bars). Bottom: Fragment size selection can increase the proportion of mutant reads. **c** Copy-number profiling. Top: Low tumor content impedes the identification of somatic copy-number alterations, as the diploid profile of wild-type DNA dilute tumor signal. Bottom: Size selection, or higher tumor content, increases the resolution of the analysis

2.1 Detection and Quantification of ctDNA

Part of the clinical utility of ctDNA lies in enabling minimally invasive monitoring of tumor levels throughout a patient's treatment, which includes the detection of minimal residual disease and the onset of relapse. These tasks require accurate measurement of the tumor fraction in blood samples. Quantification can be approached with either ultra-sensitive custom assays or general-purpose DNA sequencing. The former may achieve sensitivities up to 1 in tens or hundreds of thousands of DNA fragments, but are limited to probing up to about 100 mutations, which need to be specified in advance (Diehl et al. 2006; Taly et al. 2013). These mutations may either be hot-spots or variants that were previously identified in a patient's tumor sample. On the other hand, thousands to millions of bases can be screened for mutations by targeted sequencing methods (Forshew et al. 2012; Newman et al. 2014; Phallen et al. 2017). This strategy is especially useful for genes lacking well-defined hot-spots, such as tumor suppressors. However, the sensitivity of sequencing methods is generally limited to about 0.1–1% by factors such as the total number of cfDNA fragments, depth of sequencing, and noise from PCR and sequencing.

For sequencing approaches, the low levels of ctDNA demand PCR amplification and deep sequencing (>1000×) in order to reliably capture low-frequency mutant fragments. These processes introduce biases and artifacts that de novo mutation calling must take into consideration. Rather than simply comparing a tumor sample to its matched normal tissue, as is standard for bulk sequencing experiments, for deep sequencing of ctDNA the count of variant reads is usually tested against a panel of healthy control samples. Mutations are called if the number of variant reads observed in the test sample significantly exceeds the counts observed in the controls, suggesting that the observation is unlikely to have arisen only due to noise (Forshew et al. 2012; Newman et al. 2016). This procedure is carried out for each targeted position and each possible mutation, because noise levels differ by

genomic location and base change. To better account for artifactual mutation signatures left by PCR or sequencing, error models may consider base changes in a trinucleotide context rather than on their own. Common SNPs may be filtered using public databases, while private SNPs require a matched normal sample, usually the buffy coat of the same blood sample. Indels can be called in a similar fashion, considering position, indel type, and length as covariates. When tracking known mutations, more permissive thresholds may be used to increase sensitivity. Alternative schemes for outlier detection may be employed to distinguish true variants from background noise (Hodge and Austin 2004).

In order to improve the sensitivity of sequencing-based methods, the signal-to-noise ratio must be increased. ctDNA signal is diluted by the excess of wild-type fragments. Analysis of the fragment length distributions of wild-type and mutant fragments can identify intervals where the majority of fragments are of tumor origin. Size selection can then be applied, either experimentally or in silico, to remove fragments that have a high probability of originating from non-tumor cells (Mouliere et al. 2018). Additionally, noise can be reduced. This usually involves UMI incorporation and redundant sequencing, leading to the recognition and removal of most artifacts: Background error rates are thus lowered, increasing one's ability to call genuine variants (Phallen et al. 2017; Newman et al. 2016; Kinde et al. 2011). Endogenous barcodes, namely the alignment coordinates of a fragment, may also be used as a barcode, but care must be exercised because fragment locations are not uniformly distributed (Snyder et al. 2016). Violation of the assumption that all fragments have distinct coordinates may result in lower sensitivity.

Detection and quantification of ctDNA may also be accomplished using structural variants (SVs) and copy-number aberrations (CNAs). Targeted sequencing of SVs identified in the tumor sample provides a sensitive means of detecting ctDNA, owing to the absence of artifactual reads with the same break point (Leary et al. 2010, 2012). Instead, de novo detection of SVs requires either a sufficiently high depth of coverage or high tumor burden. CNAs can be measured using sWGS or, more coarsely, with a targeted approach (Belic et al. 2015; Heitzer et al. 2013; Adalsteinsson et al. 2017). CNA-based methods typically require a minimum of 10% tumor content to reliably distinguish low-level CNAs from background variation and may be better suited to deciding how to further analyze a patient's sample: If the tumor fraction is estimated as low, subsequent experiments will require high sensitivity and employ smaller panels; otherwise, the sample may be suitable for whole-exome or whole-genome sequencing. However, copy-number profiles derived from sWGS data should be interpreted with caution, as, in general, there is insufficient information to identify which segments are copy-number neutral. Therefore, absolute tumor copy numbers cannot be inferred, and only relative changes between segments can be quantified.

All of the above methods quantify tumor content in blood by proxy, by using either the allele fractions of SNVs, indels, and SVs, or the amplitude of CNAs. It is important to remember that these measures are functions of the clonal structure of the sample. The allele fraction of a mutation matches the tumor content of the

sample only if the mutation is homozygous, unaffected by CNAs, and if it occurred before or during the most recent selective sweep in the tumor. Generally, however, the relation between allele fractions (or copy-number segment means) and tumor fraction is more complicated and depends on the alteration under scrutiny (Marass 2016). In practice, a homogeneous tumor is often implicitly assumed, and corrections for the zygosity or prevalence of the aberrations are not applied. This problem is more acute for subclonal mutations and so less for copy-number neutral early alterations. Alternatively, tumor content could be quantified directly from the overall fragment length profile (Mouliere et al. 2018), although this may require a better understanding of the biology of fragmentation and validation across different tumor types.

2.2 Noninvasive Genome-Wide Characterization

When regular biopsies cannot be obtained, liquid biopsies may offer the only molecular description of the tumor. This problem re-occurs when the cancer evolves and one wishes to profile once again the altered genome, for example looking for resistance mutations at relapse. The ability to characterize the entire tumor genome noninvasively is thus of great importance.

Genomic characterization of the tumor genome via ctDNA can be done using broad panel sequencing, whole-exome or whole-genome sequencing. However, without a high sequencing depth, the sensitivity of this approach is low, and low-frequency mutations and clones may be missed. Analysis of multiple samples may help increase sensitivity (Josephidou et al. 2015). In general, detection is biased toward early mutations, which are shared by the majority of tumor cells (Murtaza et al. 2015). This phenomenon is simply a consequence of the accumulation of mutations in clonal evolution and plays in favor of the selection of stem mutations for immunotherapies. Calling somatic alterations may proceed as in the analysis of bulk tumor samples, comparing the ctDNA sample with a matched normal sample, or a panel of normal controls, as described above. The caveat here is the much lower tumor content. Indeed, methods designed for bulk sequencing may underperform and should be benchmarked to assess the sensitivity and specificity they provide for ctDNA data. As before, size selection may be applied to enhance tumor signal that would otherwise be too dilute, and molecular barcodes may be employed to reduce noise.

2.3 Phylogenetic Analysis of CtDNA

Recently, advancements in sequencing technology and analysis have made it possible to infer the evolutionary history of tumors from sequencing data of biopsies (Griffith et al. 2015; Nik-Zainal et al. 2012). Here, a set of mutations quantified over one or more samples is analyzed to uncover a phylogeny whose taxa are tumor clones. Even though strong signal is required to detect not only the tumor

but also its clonal composition, there are advantages to performing this analysis on ctDNA data. First, it is feasible to collect longitudinal datasets per patient. If the proportions of different clones in the circulation vary from sample to sample, more information is available to correctly distinguish the signals and assign mutations to the right clones. Second, ctDNA may better capture heterogeneity as it might be less spatially biased than needle biopsies and it collects tumor fragments from multiple lesions in the body (Chan et al. 2013; Forshew et al. 2012; Murtaza et al. 2015). Third, the short half-life [reportedly two hours (Diehl et al. 2008)] ensures that the information available in ctDNA is up to date, allowing real-time monitoring of evolution and clonal dynamics in response to treatment (Dawson et al. 2013; Siravegna et al. 2015). The main challenge for this analysis lies in the low tumor burden. High depth of sequencing and low background noise are thus needed to appreciate subclonal aberrations and to obtain accurate copy-number information, the latter being crucial to adjust the mutation allele fractions during inference. Most analyses of tumor clonality using ctDNA have relied on mutation clustering (Murtaza et al. 2015; Gremel et al. 2016; Abbosh et al. 2017), whereas a phylogenetic deconvolution was performed by Marass (2016).

3 Analysis of Circulating Tumor Cells

CTCs are tumor cells originating from cancerous lesions and found in the bloodstream. They can be found as single cells or as multicellular clusters, and can be associated with other cell types such as platelets and leukocytes (Aceto et al. 2015; Szczerba et al. 2019). In cancer patients, CTCs are extremely rare compared to the surrounding hematopoietic cells, and on average they are found at a concentration of one CTC per billion normal blood cells (Yu et al. 2011). This scarcity is the major limitation in studying CTCs, and it has spurred the development of specialized technologies for their isolation and characterization.

3.1 Capture

First-generation detection methods for CTCs were based on biological features, such as the expression of epithelial-specific markers (e.g., EpCAM or cytokeratins) that are absent in normal blood cells but are highly expressed in most tumor cells of epithelial origin (Joosse et al. 2015; Went et al. 2004). This approach is utilized in CellSearch (Riethdorf et al. 2007), currently the only FDA-approved tool for CTC enumeration from the blood of cancer patients. The CellSearch technology is based on a two-step CTC enrichment procedure, comprising EpCAM-based capture of CTCs with magnetic beads and fixation with staining for epithelial cytokeratins and the white blood cell marker CD45. However, positive selection and antigen-dependent approaches may overlook cells that express low levels of the selected markers. Moreover, CTCs captured by CellSearch are non-viable due to fixation

and thus the quality of their DNA and RNA may be compromised, affecting downstream analyses. For these reasons, antigen-independent methods that are focused on depletion of red and white blood cells, or enrichment exploiting physical features of CTCs, have been developed (Hou et al. 2013; Ozkumur et al. 2013; Sarioglu et al. 2015; Chudziak et al. 2016; Galanzha and Zharov 2013). For instance, the Parsortix technology allows antigen-independent size-based CTC enrichment through narrowing microfluidic channels at physiological flow rates, while preserving CTC integrity and viability for downstream molecular and cellular assays (Chudziak et al. 2016).

3.2 DNA and RNA Sequencing

Thanks to the development of specialized technologies for CTC isolation and characterization, it is now possible to investigate the genome and transcriptome of single and clustered CTCs. After capture, the next challenge for sequencing CTCs lies in the limited input material available from each cell. For this reason, the amplification of genome and transcriptome is a crucial step in the sequencing workflow. Multiple amplification protocols have been developed, each with specific advantages and disadvantages.

The predominant method for whole-genome amplification (WGA) from single cells is multiple displacement amplification (MDA), which utilizes the high-processivity and strand displacement features of the phi29 polymerase (Spits et al. 2006). This method is based on annealing random hexamers and constant strand synthesis, where newly synthesized DNA fragments serve as new reaction templates, resulting in an exponential amplification rate. More recently, a new method, called multiple annealing and looping-based amplification cycles (MALBACs), has been introduced. In contrast to the stable strand amplification of MDA, MALBAC is PCR-based and follows standard cycles of DNA denaturation, annealing, and extension. Limitations of the WGA process include high error rates, non-uniform coverage, complete loss of coverage for some regions, allele dropout, and allelic imbalance. Quantitative comparisons of available amplification methods have been performed; however, no single approach outperforms all others in every situation (Hou et al. 2015; Huang et al. 2015). Thus, the choice of amplification method should be tailored to the goals of each individual study (de Bourcy et al. 2014). In general, MDA shows better breadth of coverage, and it is considered the best choice for SNV analysis, due to significantly lower false-positive rates. Published studies using MDA amplification have reported exome coverage ranging from 50 to 80% in single cells (Gawad et al. 2016). MALBAC, on the other hand, leads to more uniform coverage resulting in better data for CNV profiling (Gawad et al. 2016).

Similar approaches have been developed for single-cell whole-transcriptome amplification (WTA). These methods can be categorized as PCR-based, MDA-based, or in vitro transcription (IVT) of mRNA (Van Loo and Voet 2014; Saadatpour et al. 2015). Most of these methods capture polyadenylated RNA and

can be focused on full-length transcripts or biased toward the 3′ end or the 5′ end (Van Loo and Voet 2014). The Smart-seq amplification method is among the most widely used approaches for full-length transcriptome amplification (Picelli et al. 2013). It relies on reverse transcription, template-switching oligonucleotides to anchor a primer binding site at the 3′ end of cDNA, and PCR amplification (Picelli et al. 2013). Smart-seq can achieve high coverage across transcripts, facilitating the detection of SNVs and isoforms. One limitation of this and other WTA methods is the low amplification efficiency for lowly expressed transcripts of less than ten copies per cell (Van Loo and Voet 2014).

Another important factor to consider is the extrinsic stress to which CTCs are exposed in vivo, such as hemodynamic shear forces in the bloodstream (shear stress) (Phillips et al. 2014), attacks by the host immune system, or cancer therapy. Cellular stress may result in an increased proportion of low-quality DNA and RNA material that could lead to misinterpretation of the sequencing results. For this reason, it is crucial to apply careful quality control on both genomic extraction and data analysis in order to discard low-quality cells (Ilicic et al. 2016).

3.3 Mutation and Copy-Number Calling

Despite advancements in amplification techniques, noise and the dropout of genomic intervals remain prevalent, making the detection of SNVs challenging. While computational approaches to model noise and call mutations are well developed for bulk sequencing data, robust methods for single-cell sequencing are at an earlier stage of development. In addition to missing data and allelic dropout (false negatives), each cell may also exhibit false-positive mutations due to PCR and sequencing errors. Three main methods have been developed to call SNVs in single cells. SCcaller performs variant calling correcting for local amplification bias (Dong et al. 2017). Monovar pools information across cells to compute the probability that a variant is true; mutations observed in a single cell are typically filtered (Zafar et al. 2016). A more recent tool, SCIΦ, jointly infers cellular genotypes and their phylogenetic relationship to increase sensitivity and robustness of variant calling (Singer et al. 2018).

The detection of CNVs is also affected by WGA biases. To date, three methods have been published to calculate CNVs from scDNA-seq data (Garvin et al. 2015; Knouse et al. 2016; Zhang et al. 2013). These approaches infer copy-number from GC-normalized read coverage for genomic segments defined by circular binary segmentation (Olshen et al. 2004). However, given the amplification bias and the low coverage usually obtained for single cells, the resolution of these methods is limited to the megabase scale (Garvin et al. 2015; Knouse et al. 2016). In addition, a number of studies have attempted to infer copy-number profiles from single-cell RNA sequencing (scRNA-seq) data by averaging relative expression levels over large genomic regions (Tirosh et al. 2016; Patel et al. 2014) or by using allelic imbalance (Fan et al. 2018).

3.4 Single-Cell Phylogenetics

Reconstructing the subclonal composition and mutational history of individual tumors from DNA sequencing data is a promising route toward a better understanding of tumor development, intra-tumor heterogeneity, and metastatic seeding patterns. Due to higher data availability, the majority of approaches currently rely on bulk sequencing data. However, thanks to developments in single-cell technologies, a growing number of single-cell datasets are being produced, enabling higher-resolution phylogenetic reconstructions.

Like all cells of an organism, tumor cells descend from a single ancestor, and their genealogy can be represented by a cell lineage tree, assumed to be a perfect phylogeny. This assumption is motivated by the relatively small number of mutations compared to the length of the genome, so that no genomic site is hit more than once by a mutation. Classic phylogenetic algorithms, such as hierarchical clustering, neighbor-joining, and perfect phylogeny algorithms, could be applied to single-cell data, but are ill-equipped to deal with its noise. In practice, the observed mutation profiles are generally far from their true states due to high noise levels, characterized by missing data, strongly elevated false-negative rates due to allelic dropout, and the imbalance of false-positive and false-negative rates. To address these challenges, a number of probabilistic approaches have recently been published (Kuipers et al. 2017a).

Generally, single-cell phylogenetic methods try to find the tree topology, among all possibilities, that best fits the observed data (Fig. 2). To evaluate a topology, the set of observed mutations in each cell is compared to the expected profile from the placement of the cell in the tree. If a mutation is not observed in the cell but should be present according to the tree, it is considered a false negative; conversely, if a mutation was deemed present in the cell, but its placement in the tree suggests otherwise, it is a false positive. The likelihood that the observed mutation profiles originate from the tested tree is then calculated by accumulating the probabilities of all observations of all cells. An alternative measure of fit is the posterior probability

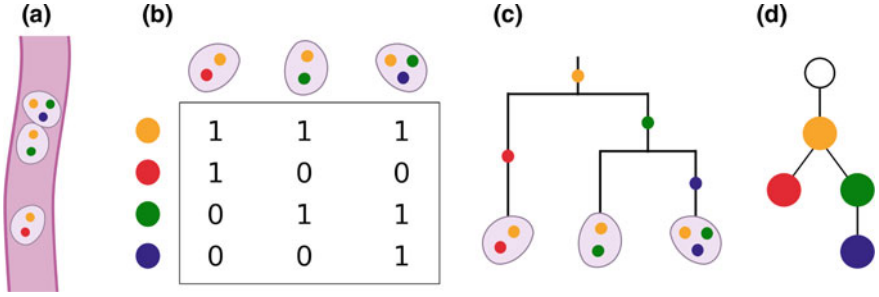

Fig. 2 Single-cell phylogenetics for CTCs. **a** Circulating tumor cells in a blood vessel, colored disks indicate mutational load of individual cells. **b** Mutation profiles of the CTCs obtained through single-cell DNA sequencing. **c** Cell lineage tree reconstructed from mutation profiles. **d** Mutation tree represents the partial temporal order in which the mutations have been acquired

of the tree given the observed data, obtained by employing Bayes' theorem. Some approaches also allow one to learn false-negative and false-positive rates from the data (Jahn et al. 2016; Ross and Markowetz 2016).

One aspect that differs across approaches is the tree model used for the reconstruction (Davis and Navin 2016). Besides the binary cell lineage tree, also known as sample tree, a clonal tree can be used that combines cells that presumably belong to the same subclone. This clustering of cells (with possibly slightly different mutation profiles) can be interpreted as an approach to correct for the high error rates of scDNA-seq data in a phylogenetic framework. Alternatively, one could consider a mutation-centric view of tumor phylogenies, namely a mutation tree. In this model, nodes are mutation events and edges represent the partial temporal order in which mutations were acquired in the tumor. To fully focus on the mutation history, this model averages out the placement of samples in the tree. Conveniently, this also evades the necessity to infer the putatively true genotypes of individual cells from the noisy scDNA-seq data, a process that is generally less reliable than the inference of the mutation history.

The first probabilistic single-cell approach was developed by Kim and Simon (2014). It uses the mutation tree model and constructs a maximum weighted spanning tree from a graph encoding the pairwise posterior probability of temporal orderings. By restricting to pairwise orderings, this method discards any higher-order relations from informing the tree inference, thus prioritizing efficiency over accuracy. BitPhylogeny is a fully Bayesian approach that uses a Markov chain Monte Carlo (MCMC) inference scheme (Yuan et al. 2015) to cluster cells with similar mutation profiles into clones. An advantage of the Bayesian framework is that the posterior distribution of trees and parameters better represents their uncertainty compared to a point estimate. However, inference can be rather costly for larger datasets.

OncoNEM uses the sample tree representation, a cell lineage tree with the sampled cells as leaves and the placement of mutations marginalized out (Ross and Markowetz 2016). The method explores the space of possible tree topologies through a greedy search with the goal of finding a maximum likelihood tree. In a second step, OncoNEM clusters similar cells to obtain a clone tree. SCITE focuses on the mutation tree representation, but can also work with sample trees (Jahn et al. 2016). The tree search is performed with an MCMC scheme that provides either a single maximum likelihood estimate or a full posterior sample to represent uncertainty in the inferred trees. Because mutation trees and sample trees can be transformed into each other, the choice of tree model can be driven by efficiency considerations: For a dataset with many mutations and few cells, the search space will be smaller under the sample tree model, while in the opposite case there are fewer candidate trees to consider under the mutation tree model.

There are a number of open challenges in tumor phylogenetics with scDNA-seq data, including the integration with bulk sequencing information (Malikic et al. 2017). A major challenge is the integration of copy-number changes into the phylogenetic reconstruction. While this shortcoming may be explained by current technological difficulties in reliably calling CNAs and SNVs from the same cells,

the integration of both data types will be an important step toward obtaining a full picture of tumor mutation histories. Another limitation is the automatic interpretation of data points incongruent with a perfect phylogeny as noise, thus ignoring the possibility of convergent evolution or the loss of mutations. However, as shown by a recent study, both events are more frequent in somatic evolution than previously thought, and ignoring them can lead to incorrect phylogenies (Kuipers et al. 2017b). Models allowing such events are starting to be developed (Zafar et al. 2017, 2018; El-Kebir 2018). Finally, none of the single-cell phylogenetics approaches are suited for handling data from multicellular CTC clusters that are sequenced as one sample. Expecting readouts to come from individual cells, existing approaches are prone to overestimating noise levels and to inferring wrong phylogenies.

3.5 Single-Cell Transcriptomics

Clustering single-cell gene expression profiles can identify subpopulations of functionally related cells. However, the available input material for scRNA-seq is very low, and not all is captured for sequencing. This creates high variability in the detected reads and dropout of expressed genes (Stegle et al. 2015). Dropout gives rise to censored data and complicates the reconstruction of the underlying expression levels. The relative uncertainty also increases with lower expression levels, thus affecting differential expression analyses. A variety of methods and statistical approaches have therefore been developed to account for these effects in scRNA-seq data (Kharchenko et al. 2014; Pierson and Yau 2015; Finak et al. 2015; Vallejos et al. 2016). Gene dropout creates a second mode in the distribution of gene expression levels, peaked around zero or low expression values, a phenomenon known as zero inflation (Kharchenko et al. 2014; Pierson and Yau 2015; Finak et al. 2015). Machine learning techniques are also starting to be employed for scRNA-seq data (Lin et al. 2017; Wang et al. 2017; Iacono et al. 2018).

In order to compare expression levels across cells or cell populations, the data need to be normalized to remove experimental confounders like the cell cycle. However, this normalization brings challenges to the analysis of single-cell experiments (Vallejos et al. 2017). Recently, single-cell-specific normalization techniques have been developed (Buettner et al. 2015; Lun et al. 2016; Qiu et al. 2017; Bacher et al. 2017; McCarthy et al. 2017). For example, latent variable models have been employed to uncover and remove confounding factors (Buettner et al. 2015). Alternatively, cells have been grouped with rank-based clustering to allow different normalization factors for each population (Lun et al. 2016), or genes have been grouped by quantile regression clustering with different scaling factors for each group (Bacher et al. 2017). A common downstream analysis of the scRNA-seq of thousands of cells has been the clustering into different cell populations (Tirosh et al. 2016a, b; Zheng et al. 2017; Patel et al. 2014; Gerber et al. 2017; Li et al. 2017; Venteicher et al. 2017), particularly to uncover new types of

cells and to explore differences in tumor populations compared to normal cells. This raises the general challenge of integrating modeling and normalization of the raw scRNA-seq data into their clustering and visualization for large datasets (Buettner et al. 2017; Risso et al. 2018).

Along with the software developed for these approaches to model and normalize scRNA-seq data, more general pipelines have been introduced and are being further developed. These include the Cell Ranger and Browser software by 10× Genomics (https://www.10xgenomics.com/software/), Seurat (http://satijalab.org/seurat/), and ASAP (Gardeux et al. 2017). These tools offer a range of standard clustering and visualization approaches and even pathway enrichment (ASAP), focusing particularly on the large datasets with tens of thousands of cells available from invasive tumor biopsies. For liquid biopsies with low cell numbers, the random effects of dropout and zero inflation and experimental confounders like the cell cycle can have a more pronounced effect on the analysis. At the same time, the computational burden falls, allowing for more detailed and intensive analyses. There is thus wider scope to build on current models so as to answer questions specific to liquid biopsies, such as identifying expression differences between single CTCs and CTC clusters.

4 Conclusion

The excitement about the field of liquid biopsies is strongly motivated by its potential to improve the detection and treatment of cancer, further our understanding of the features and vulnerabilities of invasive cancers, and provide overall benefit to the care of cancer patients. The next 5–10 years are likely to see the implementation of liquid biopsies in the clinical setting, eventually replacing invasive tumor sampling in some instances and providing a valuable additional tool for precision medicine, disease monitoring, and possibly early cancer detection. To achieve these ambitious goals, it will be necessary to implement customized computational data analysis methods based on those currently used in the research setting to interrogate ctDNA and CTCs.

The computational challenges in the analysis of DNA and RNA sequencing data obtained from liquid biopsies are manifold. They arise from the technical difficulties to capture either cell-free circulating tumor DNA or tumor cells from blood plasma in an efficient and unbiased manner, and to amplify the genomic material for sequencing uniformly. Understanding the specific patterns in the resulting short-read data will be a key for separating technical artifacts from true biological signals. If these limitations can be overcome by new experimental and bioinformatics approaches, then liquid biopsies will achieve their full potential in research and clinical practice.

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