Clinical applications of circulating tumor cells in pharmacotherapy: challenges and perspectives

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Running title: Clinical applications of CTCs in pharmacotherapy

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Number of text pages: 41

Number of tables: 1

Number of figures: 3

Number of references: 113

Number of words in Abstract: 117

Number of words in introduction: 460

Number of words in main text: 2450

Abbreviations: ALK, anaplastic lymphoma kinase; AR-V7, androgen receptor splice variant 7; CK, cytokeratins; CRPC, castration-resistant prostate cancer; CTCs, circulating tumor cells; CTM, circulating tumor microemboli; DFS, disease-free survival; EGFR, epidermal growth factor receptor; EMT, epithelial to mesenchymal transition; ER, estrogen receptor; ERCC1, excision repair cross-complementation group 1; ERK, extracellular signal-regulated kinases; HER2, human epidermal growth factor receptor 2; MET, mesenchymal to epithelial transition; MAPK, mitogen-activated protein kinases; PI3K, phosphatidylinositide 3-kinases; mTOR, mammalian target of rapamycin; PD-L1, programmed death-ligand 1
Abstract

Circulating tumor cells (CTCs) have been identified as one approach to ultrasensitive liquid biopsy for real-time monitoring of cancer patients. The detection of CTCs in peripheral blood from cancer patients is promising, but its application still faces serious challenges with respect to specificity and sensitivity. Here, we review the significant roles of CTCs in metastasis, and the strengths and weaknesses of currently available methods for CTC detection and characterization. Moreover, we discuss the clinical application of CTCs as markers for patient prognosis, and we specifically focus on the application of CTCs as indicators in cancer pharmacotherapy. Characterization of the detected CTCs will provide new biological perspectives and clinical applications for the treatment of cancer patients with metastasis.
1. Introduction

A fraction of the cancer cells that disseminate from primary tumors and migrate to distant sites will result in the formation of a lethal metastatic tumor (Hanahan and Weinberg, 2011). Migrating tumor cells found in the peripheral bloodstream are called circulating tumor cells (CTCs), while their counterparts found in bone marrow are called disseminated tumor cells (DTCs) (Alix-Panabieres and Pantel, 2016). CTCs have been regarded as a critical stage in the development of metastasis, in that they contain genetic and molecular information about the cancer as well as its evolutionary adaptation to prior therapies (Alix-Panabieres and Pantel, 2016). However, CTCs have also been detected in the blood circulation of healthy volunteers, and in patients with benign diseases of the lung, colon, pancreas, and breast (Alva et al., 2015; Cauley et al., 2015; Franken et al., 2012; Ilie et al., 2014; Liu et al., 2015; Markou et al., 2014; Pantel et al., 2012).

CTCs have paved new diagnostic avenues in liquid biopsy diagnostics, especially for tumors that are not easy to biopsy and for metastatic lesions (Azarin et al., 2015). Early detection of cancer metastasis is always difficult, not to mention its prevention or cure. With its non-invasive nature and real-time advantage, cancer screening for CTCs can be applied to populations at higher risk. Therefore, oncologists place high hopes on CTC-based screening methods which have been found to be more sensitive than current imaging methods such as PET scan (Hegemann et al., 2016). The consistency between CTCs and their primary tumors is encouraging, and may provide
an excellent opportunity for clinicians to examine mutations of key genes that are not detected through traditional blood-based assays (Nagrath et al., 2016). In the era of precision medicine in cancer therapy, systemic monitoring of response to anti-cancer therapies is a key step toward providing personalized care. An increasing body of evidence indicates that, in the course of the treatment, assessment of the molecular characteristics of the progressive disease is more significant than depending on the primary tumor samples, which do not reflect the evolution of the tumor (Alix-Panabieres and Pantel, 2016). For their comprehensive information regarding the whole disease, studies of CTCs not only reveal the underlying mechanism of tumorigenesis and metastasis, but also provide a non-invasive method for cancer diagnosis, prognosis, and pharmacotherapy monitoring (Masuda et al., 2016).

Recent research has demonstrated that CTCs, an integral part of the “liquid biopsy”, have great potential to change the status quo of anticancer therapy; however the approach remains technically challenging. Following a short discussion of the significant roles of CTCs in cancer metastasis and currently available methods for the CTCs detection and molecular characterization, this review will focus on the clinical applications of CTCs as markers for prognosis prediction in cancer patients and as indicators in cancer pharmacotherapy (Figure 1).

2. CTCs and cancer metastasis

The malignant form of cancer and the cause for more than 90% of cancer-associated
mortality is metastasis, which is characterized by the ability of cancer cells to invade into the surrounding tissue and disseminate throughout the body to establish secondary tumors in distant organs (Gupta and Massague, 2006). As shown in Figure 2, the sequential metastasis processes first initiates with a loss of adhesion of tumor cells in the primary site and their migration out of the primary tumor. Secondly, the tumor cells attach to blood vessels and invade into the blood or lymphatic circulation in a process called intravasation (Fidler, 2003). As the tumor cells circulate to the secondary site, the tumor cells intrude blood vessels to adhere to the target organ endothelium and migrate into the parenchyma; this is called extravasation (Chaffer and Weinberg, 2011; Klein, 2009; Sosa et al., 2014). Therefore, the existence of CTCs has been recognized as an important “intermediate step” in cancer metastasis. CTCs represent a stem-like sub-population of cells that are capable of immigration and tumor initiation (Al-Hajj et al., 2003; Ricci-Vitiani et al., 2007; Stewart et al., 2011). During the metastasis process, many cell surface markers of CTCs undergo change. The activation of epithelial to mesenchymal transition (EMT) facilitates tumor cell invasion and dissemination during intravasation, while its reverse process, a mesenchymal to epithelial transition (MET), is believed to support extravasation once cancer cells have arrived in distant organs (Acloque et al., 2009; Nieto, 2013; Thiery et al., 2009). It has been speculated that the entire process in which CTCs seed metastasis occurs with extremely low efficiency; only 0.01% of all CTCs can survive and form micrometastases in distant organs (Luzzi et al., 1998). Even if CTCs succeed in intravasation, most of them cannot survive the adverse environment in the bloodstream.
and eventually die from anoikis. Therefore, CTCs sometimes aggregate to form microemboli (circulating tumor microemboli, CTM), which may endow tumor cells advantages in survival and enhances CTCs viability and motility (Krebs et al., 2014).

For several decades, the lack of relevant models for metastasis research extensively limited further investigation. CTCs are the true link between primary and metastatic tumors, and thus create a new opportunity for investigators to explore valuable features of both primary and metastatic sites, as well as specific details of the processes of intravasation, migration, and extravasation.

3. CTC detection

As mentioned above, after release from the main and/or metastatic tumor site into the blood circulation, the conditions in the blood are harsh for epithelial tumor cells, and the survival time of CTCs is extremely short (half-life: 1–2.4 hours) (Meng et al., 2004). Apoptotic and fragmented CTCs are frequently detected in the peripheral bloodstream of cancer patients (Larson et al., 2004). Therefore, a pivotal challenge for the clinical application of CTCs is the capability of the current CTC technology to efficiently capture the extremely rare CTC population from patient blood samples for subsequent processing (Nelson, 2010).

In the past decade, one of the most widely used strategies to detect CTCs has been the use of epithelial markers such as cytokeratins (CK) and EpCAM, which are not detected
on the surrounding mesenchymal blood cells. CellSearch, the only U.S. Food and Drug Administration (FDA) approved CTC technology to monitor metastatic breast cancer patients, is a case in point (Ferreira et al., 2016). The CellSearch system uses ferrofluid functionalized nanoparticles containing EpCAM antibody for magnetic separation of EpCAM positive cells after blood centrifugation for the enrichment. The cells are then selected by immunostaining for the expression of CKs 8, 18 and 19, and as well as for negative staining for CD45 (Riethdorf et al., 2007).

However, further research has demonstrated that epithelial tumor cells are likely to undergo EMT, which results in decreased expression of epithelial markers and increased plasticity, migration, and invasiveness (Mani et al., 2008). These partial EMT tumor cells, also called the “intermediate phenotype,” have the highest versatility to adapt to the microenvironment in secondary sites (Tam and Weinberg, 2013). Therefore, in recent years a variety of devices have been developed for the enrichment and detection of CTCs undergoing EMT, in addition to the approaches selecting for epithelial markers (Alix-Panabieres and Pantel, 2014). CTC assays usually involves two steps: first, an enrichment step increases the percentage of CTCs, making it easier to detect single tumor cells. Specifically, CTCs can be enriched by their biologic characteristics (e.g., protein markers) or on the basis of their physical properties (e.g., size, density, deformability, or electric charges). Second, in the detection step, CTCs can be selected using different criteria such as immunologic, molecular, or functional assays (Table 1) (Ferreira et al., 2016). Nowadays, although CTC technologies have
developed rapidly, sensitivity and specificity are still problems that hinder the clinical utilization of CTCs for guiding personalized treatment of cancer patients (Hardingham et al., 2015).

4. CTCs as prognostic markers in cancer

To date, CTCs enumeration has been widely used as a prognostic index for patient overall survival rate. A cut-off value of $\geq 5$ or $\geq 3$ CTCs in 7.5 ml blood has been proved to be a poor prognostic index in several cancers, including breast cancer (Zhang et al., 2012), colorectal cancer (Cohen et al., 2008), prostate cancer (de Bono et al., 2008), lung cancer (Krebs et al., 2011), bladder cancer (Gazzaniga et al., 2014), liver cancer (Schulze et al., 2013), esophageal cancer (Vashist et al., 2012), melanoma (Rao et al., 2011), head and neck carcinoma (Nichols et al., 2012), and pancreatic cancer (Han et al., 2014).

The association between detection of CTCs and clinical outcome has been most widely studied in breast cancer. For example, CTCs were analyzed in a pool of 2026 patients with early stage breast cancer before pharmacotherapy and 1492 patients after pharmacotherapy using the CellSearch System (Rack et al., 2014). In the pre-pharmacotherapy group, CTCs were found in 21.5% of patients, in which 19.6% were lymph node-negative and 22.4% were node-positive. No correlation was found between CTCs and tumor size, grading, or hormone receptor status. CTCs were detected in 22.1% of 1492 patients after chemotherapy. The presence of CTCs was associated with poor
rates for disease-free survival (DFS), distant DFS, breast cancer-specific survival, and overall survival. CTCs were identified as an independent prognostic index for DFS in multivariable analysis. Patients with more than five CTCs per 30 mL blood had the worst prognosis. These results from a large-scale trial of patients with breast cancer suggested that CTCs have prognostic value (Rack et al., 2014).

Of note, in these reports indicating that CTCs can be used as a reliable early index of disease progression and survival as compared to traditional methods, a significant proportion of patients with obvious distant metastases were negative for CTCs. This result implied that CTCs undergoing EMT transformation can be missed by epithelial marker based detection methods, such as the CellSearch system. Therefore, large-scale multicenter trials with improved CTC detection techniques and well-defined endpoints are needed to support the clinical utility of CTC detection in cancer patients.

5. CTC as indicators in pharmacotherapy

CTCs may be disseminated from the primary tumor or from a number of metastatic sites. Therefore, CTCs offer a wealth of genetic and molecular information concerning the cancer at the protein, RNA, and genome levels (Meng et al., 2004). In addition to CTC detection, significant effort has been made towards CTC characterization. In the era of precision medicine of cancer therapy, identification of CTCs expressing certain markers can be used to specifically monitor cancer therapy.
5.1. CTCs as markers for targeted therapy selection

Molecular alterations in CTCs have proved to be highly consisted with the primary tumor, which provides robust evidence for the clinical application of targeted therapy in cancer. Several studies have suggested CTCs as an index of therapy selection and, furthermore, as a real-time biopsy to reflect the effect of a particular therapy.

For example, BRAF mutation between primary tumors and metastases have been described within a patient; these mutations mediate tumor proliferation through activation of the RAF–MEK–ERK pathway (Lin et al., 2011). Therefore, the BRAF mutation status in CTCs collected from patients with metastatic melanoma is a pivotal index for selecting targeted therapies such as Vemurafinib and Dabrafenib (Jang and Atkins, 2014; Reid et al., 2015). Another case in point are EGFR mutations in lung cancer. A group of pulmonary adenocarcinoma that have activating EGFR mutations are exclusively sensitive to EGFR tyrosine kinase inhibitors (Mok et al., 2009). Therefore, EGFR mutations in CTCs are clinical biomarkers for categorization of pharmacotherapies target in metastatic lung cancer with respect to treatment with Erlotinib, Afatinib, and Osimertinib (Breitenbuecher et al., 2014; Kuwano et al., 2016).

In addition to melanoma and lung cancer, therapeutic targets were identified in breast cancer. The PI3K/AKT/mTOR pathway is frequently altered in cancer. PI3K is a cell membrane signal transduction molecule that supports cell survival and growth, making it a popular therapeutic target (Akinleye et al., 2013; Wong et al., 2010). PIK3CA mutations were identified in CTCs from metastasis breast cancer patients by CellSearch
enrichment, DNA extraction, and whole genome amplification (Schneck et al., 2013). Agents targeting this pathway, such as Everolimus and Temsirolimus, are promising (Johnston, 2015).

Another application of CTCs is for the detection of various biomarkers expressed in advanced disease that reflect the progression of the cancer. Hormone receptor status is one of the most well-established predictors for endocrine adjuvant or palliative therapy of primary and metastatic breast cancer. However, hormone receptor status changes during the course of disease progression. Variations in the expression of ER and HER2 can occur in advanced breast cancer, and has been readily detected in CTCs. Monitoring of these changes is helpful in selecting chemotherapies, especially those targeting HER2 receptor such as Trastuzumab, Lapatinib, Pertuzumab and T-DM1 (Aktas et al., 2011; Hernández-Blanquisett et al., 2016; Thompson et al., 2010; Turner and Di Leo, 2013). In addition, several therapeutic targets such as ALK (Illei et al., 2012; Pailler et al., 2013; Pailler et al., 2013), PD-L1 (Jing et al., 2016), and RAS (Karandish and Mallik, 2016), were also detected in CTCs collected from breast, colorectal, prostate and ovarian cancer patients (Figure 3).

5.2. CTCs as markers of treatment resistance

It has been reported that, in both early and metastatic cancers, the presence of CTCs following treatment can act as a predictive index of the possibility of disease recurrence (Alix-Panabieres and Pantel, 2013; Xenidis et al., 2007). These persisting CTCs are
resistant to treatment and can thus be involved in cancer progression. Therefore, there is an urgent need to identify effective therapies in patients with “therapy-resistant” CTCs. Several studies have been reported based on this strategy. For example, platinum resistance is one of the most recognized clinical challenges for ovarian cancer pharmacotherapy. While detection of excision repair cross-complementation group 1 (ERCC1) protein in the primary tumor by immunohistochemistry is inaccurate for the prediction of platinum resistance, the presence of ERCC1(+) CTCs in blood can be used as a diagnosis biomarker in ovarian cancer to predict platinum resistance (Kuhlmann et al., 2014). In metastatic castration-resistant prostate cancer (CRPC), the presence of androgen receptor splice variant 7 (AR-V7) in CTCs is associated with resistance to enzalutamide or abiraterone, but not to taxanes (Antonarakis et al., 2015; Antonarakis et al., 2014). In AR-V7-positive patients, Taxanes are more efficacious than enzalutamide or abiraterone therapy in AR-V7-positive tumors, while in AR-V7-negative, taxanes, enzalutamide, and abiraterone have quite similar efficacies (Antonarakis et al., 2015). AR-V7 expressed in CTCs may therefore serve as a biomarker for CRPC treatment selection (Onstenk et al., 2015). These results add to existing evidence that CTCs are a valuable tool to optimize personalized cancer treatments and improve the prognosis for therapy-resistant patients.

5.3. CTC as a biomarker for treatment sensitivity

Increasing evidence points out the significance of evaluating molecular features of the advancing disease during therapy, instead of depending on the primary tumor sample
that are unable to reflect the progression of the tumor and target associated features (Alix-Panabieres and Pantel, 2016). Considering the easy availability of blood, it has been suggested that CTCs can serve as a “real-time liquid biopsy” to provide information of the current disease without invasive biopsy (Lianidou and Markou, 2011).

CTC enumeration is one of the most widely-used criteria to monitor systemic anticancer therapy. The significance of CTC enumeration in monitoring anticancer therapy has been demonstrated in metastatic breast cancer patients receiving first-line chemotherapy. In the SWOG 0500 (NCT00382018) clinical trial, patients with metastatic breast cancer had CTC enumeration before cycles 1 and 2 with or without targeted therapy in combination with first-line chemotherapy. Patients with sustained increasea in CTC number (≥5 CTCs/7.5 mL) after one cycle treatment were regarded as at higher risk group for early cancer progression. These patients were randomly designated into a continued first-line pharmacotherapy group or another treatment group before radiologic evaluation of progression (Bidard et al., 2016). For patients with continued increases in CTC numbers after first-line chemotherapy, a more effective treatment than standard chemotherapy is needed (Smerage et al., 2014). Several multicenter clinical trials testing anticancer therapy monitoring based on CTCs are still in progress, including the STIC CTC METABREAST clinical trial in France (NCT01710605). In this trial, breast cancer patients with more than 5 CTC counts in 7.5 mL blood received chemotherapy, while patients with no more than 5 CTCs in 7.5
mL blood received endocrine therapy as the first-line treatment (Lianidou and Markou, 2011). In a phase II trial of Erlotinib and Pertuzumab in advanced non-small cell lung cancer, CTC counts were associated with treatment response rates, which were correlated with fludeoxyglucose-positron emission tomography (Punnoose et al., 2012).

6. Future prospect and challenges

There is no doubt that innovative approached utilizing CTCs have paved new diagnostic avenues for the next generation of liquid biopsy diagnostics, especially in tumors that are not easy to biopsy and in metastatic lesions. Furthermore, based on their non-invasive and real-time advantages, CTCs can be applied for cancer screening of populations at higher risk. Identification and characterization of CTCs have been applied in several key clinical areas, such as prognosis prediction, systemic pharmacotherapy selection, and monitoring. However, although some promising results have been reported, detection of CTCs still faces serious challenges including sensitivity and specificity. In the future, more efficient capture systems and larger panels of detection markers will be explored to avoid losing assay specificity while increasing sensitivity. In conclusion, the detection and characterization of CTCs will provide new biological perspectives and clinical implications for cancer patients, especially during pharmacotherapy.

Authorship contribution

Wrote or contributed to the writing of manuscript: Wu, Cheng, and Fu.
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Footnotes

This work was supported by grants from National Nature Scientific Foundation [No. 81473233, 81600878], International Collaboration Science Research Foundation of Guangdong Province [No. 2013B051000046], Medical Science and Technology Research Fund of Guangdong Province [No. B2013145], Natural Science Foundation of Guangdong Province [No.2016A030310217] and Medical Scientific Research Foundation of Guangdong Province [No.A2016096].
Figure legends

Figure 1. Clinical application of CTCs as liquid biopsy. Recent research has demonstrated that CTCs, an integral part of the “liquid biopsy”, have great potential to change the status quo of cancer therapy. One of the most commonly used clinical applications of CTCs is as markers for cancer patient prognosis prediction based on CTC enumeration. Other categories of CTC clinical application are as indicators in cancer pharmacotherapy such as markers of targeted therapy selection, treatment resistance and sensitivity.

Figure 2. Schematic representation of the participation of circulating tumor cells (CTCs) in multiple stages of metastasis. The sequential metastasis process initiates with a loss of adhesion of tumor cells in the primary site and their migration out of the primary tumor. Next, the tumor cells attach to the blood vessels and invade into the blood or lymphatic circulation, which is called intravasation. The activation of epithelial to mesenchymal transition (EMT) facilitates tumor cell invasion and dissemination during intravasation. CTCs sometimes aggregate to form microemboli (circulating tumor microemboli, CTM), which may endow tumor cells with advantages in survival and enhances their viability and motility. As the tumor cells circulate to the secondary site, they intrude blood vessels to adhere to the target organ endothelium and migrate into the parenchyma, which is called extravasation. Activation of mesenchymal to epithelial transition (MET) is believed to support this extravasation once cancer cells have arrived in distant organs.
Figure 3. Chemotherapeutic targets identified in circulating tumor cells (CTCs) and their representative target agents. Several therapeutic targets such as EGFR, HER-2, ALK, PD-L1, RAS were detected in CTCs collected from lung, breast, colorectal, prostate and ovarian cancer. These proteins are clinical biomarkers for target therapy selection.
<table>
<thead>
<tr>
<th>Enrichment</th>
<th>Selection criteria</th>
<th>Assay system</th>
<th>Tumor origin</th>
<th>Key feature</th>
<th>Reference</th>
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<tr>
<td>Immunoaffinity-positive</td>
<td>EpCAM</td>
<td>CellSearch®</td>
<td>Colorectal, breast, prostate, ovarian, lung</td>
<td>FDA approved</td>
<td>Ref. (Ohnaga et al., 2016), (Grisanti et al., 2016; Shaw et al., 2016; Sholl et al., 2016; Van Berckelaer et al., 2016)</td>
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<td></td>
<td>MagSweeper</td>
<td>Breast and prostate</td>
<td>High purity</td>
<td>Ref. (Cann et al., 2012; Deng et al., 2014)</td>
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<td>CTC-Chip</td>
<td>Breast, esophageal, prostate, lung</td>
<td>Micro-post array</td>
<td>Ref. (Jiang et al., 2015; Khamenehfar et al., 2015; Ohnaga et al., 2016; Sequist et al., 2009)</td>
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<td>GEM</td>
<td>Pancreatic</td>
<td>Micro-vortices sample mixture</td>
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<td>Antibody cocktail</td>
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<td>AdnaTest</td>
<td>Breast, colorectal, ovarian, prostate</td>
<td>Multiple cancer marker measured by RT-PCR</td>
<td>Ref. (Blassl et al., 2016; Bredemeier et al., 2016; Capoun et al., 2016; Gorges et al., 2016)</td>
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<td>LiquidBiopsy</td>
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<td>Breast</td>
<td>Automated, sheath</td>
<td>Ref. (Winer-Jones et al.,</td>
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<td>Method</td>
<td>Cells Separated</td>
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<td>Immunoaffinity-negative</td>
<td>CD45, CD66b, size</td>
<td>Flow minimized non-specific binding continuous flow</td>
<td>(Karabacak et al., 2014; Ozkumur et al., 2013)</td>
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<td></td>
<td>CTC-iChip</td>
<td>Size-based separation debulks whole blood, inertial focusing aids in magnetic deflection</td>
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<td>Density gradient centrifugation</td>
<td>Density Enrichment and CyteSealer</td>
<td>Sequential density fractionation enriches target cells.</td>
<td>(Campton et al., 2015)</td>
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<td></td>
<td>Accucyte</td>
<td>(Lung, prostate, pancreas, breast, melanoma)</td>
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<td>Microfiltration in two and three dimensions</td>
<td>Size, deformability deformability</td>
<td>Sensitive threshold of one carcinoma cell per milliliter of blood.</td>
<td>(Abdallah et al., 2016; Li et al., 2015; Long et al., 2016; Pailler et al., 2015)</td>
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<td>ISET®</td>
<td>(Colorectal, melanoma, esophageal, pancreatic, lung)</td>
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<td>Electrophoresis</td>
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<td>Continuous flow, captures viable cells.</td>
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<td>Inertial focusing</td>
<td>Size Vortex</td>
<td>Microfluidic technology for the label-free, size-based enrichment and concentration of</td>
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<td>Size Acoustophoresis Chip.</td>
<td>Breast</td>
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<td>Ref. (Catenacci et al., 2015; Dent et al., 2016; Lopez-Riquelme et al., 2013; Starzynska et al., 2013)</td>
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<td>Cell adhesion matrix Vita-Assay™</td>
<td>Prostate, lung, pancreatic</td>
<td>Ref. (Friedlander and Fong, 2014; Tulley et al., 2016)</td>
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Figure 1

CTCs as prognostic markers in cancer

CTCs enumeration: a cut-off value of ≥5 or ≥3 CTCs in a 7.5 ml blood sample has been shown to be a poor prognostic marker

Markers of targeted therapy selection

Markers of treatment resistance

Markers of treatment sensitivity

CTCs as chemotherapy indicators in cancer

Markers of treatment response rates, which were correlated with PET Response Evaluation Criteria In solid tumor

ERCC1(+): Platinum resistance in ovarian cancer

AR-V7(+): Enzalutamide or abiraterone resistance in CRPC

Markers of targeted therapy selection

EGFR mutation: Afatinib etc.
BRAF mutation: Vemurafenib etc.
PIK3CA mutation: Everolimus etc.
HER2(2+): Pertuzumab etc.
PD-L1(+): Nivolumab etc.
Ras(V): Tipifarnib etc.
ALK(V): Crizotinib
Figure 3

CTC