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Circulating tumor cells: advances in isolation and analysis, and challenges for clinical applications

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Abstract

Circulating tumor cells (CTCs) are rare cancer cells released from tumors into the bloodstream that are thought to have a key role in cancer metastasis. The presence of CTCs has been associated with worse prognosis in several major cancer types, including breast, prostate and colorectal cancer. There is considerable interest in CTC research and technologies for their potential use as cancer biomarkers that may enhance cancer diagnosis and prognosis, facilitate drug development, and improve the treatment of cancer patients. This review provides an update on recent progress in CTC isolation and molecular characterization technologies. Furthermore, the review covers significant advances and limitations in the clinical applications of CTC-based assays for cancer prognosis, response to anti-cancer therapies, and exploratory studies in biomarkers predictive of sensitivity and resistance to cancer therapies.

Keywords

circulating tumor cells; CTC; cancer biomarker; prognostic biomarker; predictive biomarker

1. Introduction

The process of cancer metastasis by which tumor cells detach from a primary site, spread through the circulatory system, and form distant secondary tumors is responsible for the majority of cancer deaths (Hanahan and Weinberg, 2011). Since they were first described by Thomas Ashworth in 1869, the presence of circulating tumor cells (CTCs) has been suggested to be associated with cancer by various early studies (Ashworth, 1869; Carey et al., 1976; Gallivan and Lokich, 1984; Myerowitz et al., 1977). Through a proposed process known as epithelial-mesenchymal transition (EMT), epithelial cells of solid tumors undergo cellular changes that enable them to escape their structural confines via increased mobility and invasiveness, to enter into the blood stream, and to adhere and develop into distant metastases (Steeg, 2003; Thiery, 2002). Thus, it is very attractive to isolate and characterize CTCs, as they may represent both the phenotypic and genetic compositions of the primary tumors and potentially serve as a “liquid biopsy” for any metastatic tumors.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

A validated CTC enrichment and enumeration technology has been established in which CTC counts above a known threshold is a prognostic marker and predictor of patient outcome in metastatic breast (Hayes et al., 2006), prostate (Danila et al., 2007), and colon cancers (Cohen et al., 2008). Based on these clinical trials, the US Food and Drug Administration (FDA) cleared the CellSearch® technology (Veridex, LLC, Raritan, NJ, USA) for CTC enrichment and enumeration for the above indicated cancers. The success of CellSearch® proves that enumeration of CTCs is indeed a surrogate for active disease and that increased CTC numbers are predictive of worse prognosis. Also, by demonstrating the successful isolation of clinically relevant cells from the blood of cancer patients, it revealed the potential for further analysis of CTCs beyond enumeration.

There is great interest in obtaining molecular information from CTCs, as they may constitute a read-out for both primary and metastatic tumors. Success in CTC-based analysis has the potential to provide real-time and non-invasive surrogates for diagnosis and prognosis, predictive biomarkers for making treatment decisions, and samples for monitoring drug resistance. The majority of conventional cancer treatments have had limited success in curing metastatic disease. As tumors evolve, even an effective response to therapy is typically short lived, and patients often relapse within 12-24 months of therapeutic intervention (Cristofanilli et al., 2005; Lacy et al., 1998; Ushijima, 2009). CTCs may provide a source for longitudinal molecular analysis of tumors during the clinical management of patients that could facilitate both clinical investigations and cancer patient care.

The fact that CTCs occur at extremely low levels in the circulation and are obscured by billions of peripheral blood cells has hindered their isolation and molecular characterization (Alix-Panabières and Pantel, 2013). There have been numerous efforts, and many technologies developed to enrich and analyze CTCs, many of which have been explored and evaluated with samples from cancer patients. This review will mainly focus on CTC enrichment technologies, studies, and applications that have been successfully tested or evaluated with clinical samples. We will review the recent advances that have been made towards applying CTC assays to clinical practice, discuss the substantial challenges facing the field, and elaborate on future prospects.

2. Isolation of CTCs: current advances

CTC isolation techniques must be sensitive enough to capture the rare and heterogeneous population of CTCs, while also being sufficiently specific for substantial enrichment against blood cells. It is also important for the isolation to be repeatable, reliable, rapid, cost-effective, capable of processing clinically-relevant volumes of blood, and compatible with process automation and downstream CTC analysis. Further, it is desirable for some analyses that isolated cells will maintain their viability and that they experience minimal disturbance caused by the isolation process that might alter their status or phenotype.

Various approaches that have been developed for CTC isolation from blood are discussed below. The technologies are grouped by their principle of CTC enrichment as illustrated in Figure 1, and summarized in Table 1. These technologies are typically evaluated using cell line model systems for multiple performance parameters (i.e. capture efficiency/recovery, enrichment against leukocytes, cell viability, processing speed, blood sample capacity) and then validated through testing with clinical samples. The optimal isolation approach may require a compromise among performance parameters, and is likely to depend on the intended downstream application.

Due to differences in the underlying principles of isolation, the cells acquired by different methods are likely to be overlapping CTC subpopulations. Thus, it is important to fully

characterize isolated CTCs and to establish clinical correlation and usability as in the case of the CellSearch® clinical trials. It is also important to compare various CTC isolation approaches to fully appreciate the benefits and drawbacks of each method. Practically, this may be achieved through blind comparison with the CellSearch® instrument using duplicate clinical samples.

2.1. Immunoaffinity

Immunoaffinity-based CTC isolation takes advantage of highly specific affinity reactions between capture antibodies and target antigens present on cells of interest. The following antibody capture approaches have been developed:

2.1.1 Magnetic beads—The CellSearch® instrument is the only FDA-cleared technology that is clinically applied for CTC enrichment. Its enrichment process involves the binding of antibody functionalized magnetic beads to the epithelial cell adhesion molecule (EpCAM) antigen on CTCs, and the subsequent isolation of these beads with a magnet. Enumeration of CellSearch®-enriched CTCs has been established as a prognostic marker and predictor of patient outcome in metastatic breast (Hayes et al., 2006), prostate (Danila et al., 2007), and colon cancers (Cohen et al., 2008). A similar approach has been developed using a prototype magnetic sweeper device to improve the capture of cells bound to anti-EpCAM-coated magnetic beads. This technology has demonstrated a capture efficiency of 62%, a purity of 51% from whole blood, and a throughput of 9 mL/hour (Talasaz et al., 2009). This "MagSweeper" device identified CTCs in 14 of 20 primary and 21 of 30 metastatic breast cancer patient blood samples using single cell level reverse transcription polymerase chain reaction (RT-PCR) based detection (Powell et al., 2012). A recent magnetic sifter device generates extremely high magnetic field gradients around the edges of magnetic pores in a microarray format to enhance capture efficiency to 91.4% or higher and performs enrichment in a vertical flow configuration to improve the processing speed, optimized at 10 mL/hour (Sieuwerts et al., 2009). AdnaTest® (Adnagen AG, Langenhagen, Germany) is a commercialized series of assays that employs magnetic beads functionalized with cocktails of antibodies specific to either breast, prostate, colon, ovarian or EMT/stem cell markers to improve enrichment. AdnaTest BreastCancer™ coupled with multiplexed RT-PCR based CTC detection has been demonstrated to correlate with patient outcome in metastatic breast (Tewes et al., 2009) and ovarian cancer (Aktas et al., 2011a). The assay was also used to identify a subset of CTCs expressing stem cell and EMT markers in primary breast cancer (Kasimir-Bauer et al., 2012). In a comparative study AdnaTest BreastCancer™ was positive for 29 of 55 metastatic breast cancer patients compared with the detection of ≥2 CTCs in 26 of 55 patients by CellSearch® (Andreopoulou et al., 2012).

2.1.2 Microfluidic flow—Nagrath, Toner and colleagues developed a microchip consisting of an array of 78,000 silicon micropillars functionalized with antibodies targeting EpCAM, allowing the direct processing of whole blood. The micropillar geometry provides an abundant total surface area for potential contact (970 mm^2), resulting in a capture efficiency of ≥60% and a final sample purity of about 50% when processing at a throughput of 2.5 mL/hour. This "CTC-chip" was used to enrich and identify CTCs in 115 of 116 tested blood samples from patients with various metastatic cancer types (Nagrath et al., 2007). A similar micropillar approach was implemented on a "geometrically enhanced differential immunocapture" microchip coated with antibodies targeting prostate-specific membrane antigen (PSMA). This microchip achieved a capture efficiency of 85% and purity of 68%, and identified CTCs from 18 of 20 prostate cancer patient samples (Gleghorn et al., 2010). To overcome fabrication challenges with the first generation CTC-chip, Stott and Toner et al have reported an improved second generation "herringbone chip" that encouraged microfluidic mixing through the generation of microvortices (Stott et al., 2010a). Here the

specific capture antibodies were conjugated to the herringbone-shaped grooves along the bottom surface of the device, and the flow patterns resulted in increased cell to surface contact. This improved capture efficiency to 91.8% using antibodies for EpCAM, and CTCs and microclusters were detected in samples from 14 of 15 prostate cancer patients (Stott et al., 2010a). More recently, to facilitate the retrieval of CTCs for further analysis, Ozkumur and Toner et al developed a "CTC-iChip" that enables either positive anti-EpCAM CTC selection or leukocyte depletion after an initial size-based enrichment step and hydrodynamic focusing. They reported high capture efficiencies of up to 98.6%, with varying purities in the range of 0.02 – 42%. CTCs were detected in 37 of 42 metastatic cancer patient samples as compared with 29 of 42 using CellSearch® (Ozkumur et al., 2013).

2.1.3 Nanostructured substrate—Wang et al employed nanostructured substrates to take advantage of an extremely high contact surface area for immunoaffinity due to roughness at the nano-scale (Wang et al., 2009). They used anti-EpCAM conjugated silicon nanopillars and chaotic micromixing to achieve a capture efficiency of >95% from blood at an optimal throughput of 1 mL/hour. This approach detected CTCs in 20 of 26 prostate cancer patient blood samples as compared with only 8 of 26 using the CellSearch® system (Wang et al., 2011). The chip was further modified with electrospun polymer nanofibers and incorporated with laser capture microdissection to isolate single prostate cancer CTCs for amplification and whole exome sequencing (Zhao et al., 2013).

2.1.4 Microtubes—Hughes et al employed a biomimetic approach to simulate the process of selectin-mediated cell adhesion in blood vessels for CTC capture. Selectin-coated microtubes induce cell attachment and rolling, which encourages CTC binding with anti-EpCAM and anti-PSMA antibodies, even at a high flow rate of 4.8 mL/hour. The device achieved a capture efficiency of ~50% and an average purity of 66%, while successfully detecting CTCs in 14 of 14 tested patient blood samples as compared with 9 of 14 with the CellSearch® system (Hughes et al., 2012).

2.1.5 *In vivo* sampling—A novel approach for *in vivo* sampling was developed by functionalizing a medical wire with EpCAM antibodies. The medical wire is injected through a cannula into the patient's cubital vein for a duration of 30 minutes to allow direct continuous sampling of large volumes of blood (1.5 - 3 liters). This approach successfully enriched CTCs in 22 of 24 patients diagnosed with either breast or lung cancer (Saucedo-Zeni et al., 2012).

2.1.6 Leukocyte depletion—An alternate approach to positive immunoaffinity based CTC selection is to use monoclonal antibodies targeting leukocyte antigens (i.e., CD45, CD14) to deplete cells of hematopoietic origin. Some strategies include antibody labeling of leukocytes for removal through immunomagnetic separation (Lara et al., 2004; Yang et al., 2009), or through centrifugation with the RosetteSep™ kit (StemCell Technologies, Vancouver, Canada) (Baccelli et al., 2013; He et al., 2008). These approaches are capable of high recovery rates with minimal disturbance to CTCs, but may achieve relatively low sample purities.

Summary—The specificity of the antibody-antigen reaction allows the isolation of CTCs with a very high level of purity. However, CTC enrichment results will be heavily dependent on the performance of the particular antibody employed. There is currently no known ideal CTC antigen target that would allow capture of all CTCs at the exclusion of all hematopoietic cells. Recent reports have indicated that anti-EpCAM approaches may miss out on significant populations of CTCs that do not exhibit an epithelial phenotype,

presumably due to EMT, in addition to the variable expression of EpCAM on certain phenotypes of epithelial CTCs (Sieuwerts et al., 2009; Yu et al., 2013). Capture rates may be increased by using combined cocktails of antibodies that also target antigens specific to a particular cancer type, though this may then result in reduced specificity and lower sample purity. Leukocyte depletion approaches have the benefit of not disturbing the CTCs which may minimize phenotypic alterations caused by the isolation process, but may lose CTCs that are attached to or interacting with leukocytes. Leukocyte depletion methods result in purities that are typically at least one order of magnitude lower than positive CTC targeting. In general, an immune-affinity approach requires long incubation/interaction times to optimize CTC recovery, which could be a bottleneck for processing speed. For methods using positive selection, it might be challenging to reversibly remove CTCs from the immune-affinity tag or surface.

2.2. Physical properties

Physical properties may be exploited to effectively separate CTCs from peripheral blood cells. The following technologies have been developed based on differences in density, size, deformability and electrical properties:

2.2.1. Density gradient centrifugation—Centrifugation is a cheap and efficient method for separating CTCs in the mononucleocyte fraction of blood away from erythrocytes and granulocytes based on cell density. Centrifugation with Ficoll-Paque® solution (Pharmacia-Fine Chemicals, Uppsala, Sweden) was used to detect CTCs using an RT-PCR based assay for cytokeratin 20 expression with a resolution of 1 cell/mL of blood in model systems, successfully identifying CTCs in 24 of 58 colorectal cancer patients undergoing surgical resection (Weitz et al., 1998). OncoQuick® (Grenier BioOne, Frickenhausen, Germany) is a novel technology that incorporates a porous barrier for size-based separation of CTCs in conjunction with density-based centrifugation. Rosenberg et al reported a vastly improved enrichment of 632-fold against leukocytes with OncoQuick® compared to 3.8-fold with Ficoll-Paque® (Rosenberg et al., 2002). OncoQuick® has identified CTCs in blood samples obtained from 11 of 37 gastrointestinal cancer patients with RT-PCR (Rosenberg et al., 2002), 5 of 60 primary breast cancer patients and 25 of 63 advanced breast cancer patients by immunofluorescence (IF) (Muller et al., 2005). In another clinical study CTCs were detected in 14 of 61 patients using cytospins prepared after OncoQuick® enrichment, compared to 33 of 61 with CellSearch® (Balic et al., 2005). Recently, leukapheresis has been shown as a centrifugation technique to sample a much larger volume of patient blood for CTC analysis, resulting in improved sensitivity for downstream detection and analysis. CTCs were detected in 21 of 29 carcinoma patient samples using the CellSearch® system after concentration by leukapheresis, compared with only 8 of 29 using the conventional 7.5 mL assay (Fischer et al., 2013).

2.2.2. Microfiltration—Microfiltration operates on the principle of retaining larger CTCs while allowing smaller leukocytes to pass through pores of varying geometries. Vona, Paterlini-Brechot, and colleagues developed the “isolation by size of epithelial tumor cells” (ISET) technique using randomly track-etched polycarbonate filters with 8 μm diameter circular pores for CTC enrichment and cytological detection from fixed blood samples (Vona et al., 2000). Track-etched microfilters have been used to enrich and characterize CTCs in studies involving liver cancer (Vona et al., 2004), melanoma (De Giorgi et al., 2010), lung cancer (Hofman et al., 2011a; Lecharpentier et al., 2011), prostate cancer (Chen et al., 2012) and various other cancers (Hofman et al., 2011b). These microfilters were demonstrated to be more sensitive than CellSearch®, detecting CTCs in 57 of 60 metastatic patients with breast, prostate and lung cancer compared to 42 of 60 with CellSearch® (Farace et al., 2011). Zheng, Tai, and colleagues used deterministic photolithography to

develop an improved microfilter with circular- or oval-shaped pores fabricated from parylene polymer, reporting a capture efficiency of 89% (Zheng et al., 2007). This parylene microfilter obtained CTCs in 51 of 57 cancer patients while CellSearch® was positive for only 26 (Lin et al., 2010). Similar microfilters have been developed from silicon substrates (Lim et al., 2012) and electroformed nickel (Hosokawa et al., 2010) detecting CTCs in 37 of 42 lung cancer patient samples compared with 19 of 43 with CellSearch® (Hosokawa et al., 2013). To encourage viable CTC capture a three-dimensional microfilter was designed out of two layers of parylene to incorporate support structures that mitigate cell damage (Zheng et al., 2011). Xu et al. employed a slot pore microfilter designed by Tai's group to detect telomerase activity from viable enriched CTCs filtered from Ficoll-Paque®-isolated buffy coats of metastatic prostate cancer patients (Xu et al., 2010).

2.2.3. Microfluidics—Microfluidic devices have been developed to achieve size and deformability-based sorting of CTCs in a more controlled fashion. Tan, Lim, and colleagues designed crescent-shaped trap arrays with a fixed 5 μm gap width to enrich CTCs from whole blood, reporting a capture efficiency and purity of over 80% (Tan et al., 2009a). This microdevice successfully detects CTCs in 1-3 mL blood samples obtained from metastatic lung cancer patients (Tan et al., 2010). Higher throughput microfluidic approaches apply hydrodynamic forces to select for cells of different sizes by inertial flow fractionation. This principle was incorporated through contraction and expansion reservoirs developed for pinch alignment of tumor cells by Bhagat et al (Bhagat et al., 2011) and tumor cell trapping in microscale vortices by Hur et al (Hur et al., 2011). An improved form of the latter approach has been applied to detect cancer cells from a cohort of 12 breast and lung cancer patients (Sollier et al., 2013). These devices allow vastly improved throughput and are capable of processing larger sample volumes compared to previous microfluidic approaches, but with potential reductions to cell recovery rate and enrichment against leukocytes. Sun et al developed a double spiral microfluidic channel to hydrodynamically separate tumor cells using drag forces, reporting a recovery rate of 88.5% from diluted blood (Sun et al., 2012; Sun et al., 2013). Lim and colleagues incorporated a spiral microfluidic channel to successfully enrich CTCs and microclusters from 20 metastatic lung cancer patients (Hou et al., 2013).

2.2.4. Dielectrophoresis—Electrical properties of CTCs may be exploited to discriminate them from blood cells by applying a non-uniform electric field through the phenomenon of dielectrophoresis (DEP). Interdigitated gold electrodes were used by Becker, Gascoyne, and colleagues to isolate leukemia (Becker et al., 1994) and breast cancer cell lines (Becker et al., 1995) from spiked healthy donor blood. The application of an electric field generated by the electrodes attracts tumor cells by positive DEP, while other cells flow past. Upon removal of the electric field the tumor cells can be collected with a capture efficiency of 95% (Becker et al., 1995). Based on the success of this method, Huang et al proposed a DEP field flow fractionation approach to allow continuous processing of samples that did not require intermittent application of the electrical field for cell recovery (Huang et al., 1997). Gupta et al developed the ApoStream™ instrument for DEP field flow fractionation, demonstrating viable capture with an efficiency of greater than 70% from cell lines spiked into whole blood (Gupta et al., 2012). Thus far, only preliminary efforts of the application of this technology to clinical samples have been reported (Shim et al., 2013).

Summary—Physical separation is achieved through simple approaches that isolate CTCs independent of cell antigen expression. They are therefore not vulnerable to antigen expression variability or loss of epithelial markers that may occur due to EMT, and typically achieve high capture efficiencies of greater than 80%. Density gradient centrifugation is a robust technique that is very effective as a first enrichment step. Leukapheresis is a

promising yet somewhat more invasive approach that allows the sampling of much more blood, improving detection sensitivity in cancer patients. However, density based approaches result in insufficient purity for most downstream analyses and typically require further enrichment. Microfiltration enables extremely high throughput processing of full tubes of blood within minutes. However, the overlap in size distributions between CTCs and large leukocytes results in sample purities of less than 10%. It is also possible that smaller CTCs or CTC fragments may be missed. Improved control in microfluidic chambers can achieve high sample purities of greater than 80%, but at the expense of reducing throughput to approximately 1 mL/hour. Higher throughputs may be achieved using hydrodynamic separation in microfluidic channels, but with reduced capture efficiency and/or purity. The use of DEP to exploit cell electrical properties is a novel approach that has demonstrated excellent viability and minimal disruption to captured cells in testing with cell lines. DEP based technology has yet to be thoroughly evaluated with clinical samples, so its clinical utility must be determined.

2.3. Direct analysis

An alternative approach to overcoming the challenges of isolating rare CTCs is to carry out high-throughput assaying of the entire population of cells in blood.

2.3.1. Fiber-optic array scanning—Krivacic, Kuhn, and colleagues developed a high-throughput “fiber-optic array scanning technology” system capable of analyzing 300,000 cells per second (Krivacic et al., 2004). Whole blood is first treated for erythrocyte lysis, and then all remaining cells are plated onto several glass slides. This approach has demonstrated efficient enumeration of CTCs in breast, prostate, pancreatic, lung and ovarian cancers (Cho et al., 2012; Lazar et al., 2012; Marrinucci et al., 2012; Nieva et al., 2012; Phillips et al., 2012; Wendel et al., 2012).

2.3.2. Micro-Hall sensor—Issadore et al used microfabricated Hall effect sensors to detect CTCs with an assay throughput of ~10^7 cells/min. CTCs are labeled with magnetic nanoparticles conjugated to antibodies targeting various antigens of interest (ie. EpCAM, HER2, EGFR, MUC1), then flowed past an array of sensors that measure Hall voltages induced by the magnetic flux of each labeled cell. This approach demonstrated improved sensitivity over the CellSearch® system when evaluated with a cohort of 20 ovarian cancer patients (Issadore et al., 2012).

Summary—Direct analysis approaches are promising for CTC detection as they are less vulnerable to cell loss. They involve minimal enrichment of blood samples, typically only requiring erythrocyte lysis. Fiber-optic scanning has enabled a “high definition-CTC” assay that allows effective CTC detection with particularly high quality imaging and optical analysis. The lack of concentration of CTCs or enrichment against leukocytes does however pose challenges for other desirable CTC analysis applications. The development of simple on-chip electrical detection with Hall effect sensing does not require optical instrumentation and therefore has great potential for portability and point-of-care application, though the approach is essentially limited to CTC enumeration.

3. Analytic tools for CTCs

The successful clinical application of CTCs requires the development of analytic tools to exploit their potential for affecting clinical outcomes. Various analytic tools have been deployed for the cellular and molecular characterization of CTCs (Figure 2). These tools are developed in conjunction with CTC enrichment technologies as each downstream application will have its own requirements regarding sensitivity, sample purity, cell

viability, and ability to recover cells after enrichment. Different isolation technologies may, therefore, be more suitable for specific downstream applications. As any CTC isolation and analysis process applies certain criteria for enrichment and detection, it is important to characterize the false-positive and false-negative rates of the overall CTC process, which can be performed with cell line models. For clinical samples, a common practice to establish a new method is to compare results from duplicate samples with the CellSearch®.

3.1. Immunophenotyping

Immunostaining is the current gold standard technique for CTC detection and enumeration as established by the FDA-cleared CellSearch® system. The defined criteria for identifying CTCs includes (1) positive expression of cytokeratins (CK), a class of intermediate filaments in epithelial cells; (2) negative expression of the leukocyte common antigen, CD45; and (3) positive staining of the nucleus with DAPI (Cristofanilli et al., 2004). Markers specific to cancer types, such as PSA or PSMA in prostate cancer, have also been used for positive CTC identification (Kirby et al., 2012; Stott et al., 2010b; Wang et al., 2000).

Recently, attempts have been made to investigate the expression of other proteins to further characterize CTCs. The presence of stem cell markers, such as CD44+/CD24−, CD133+, ALDH1+, have been identified in CTCs (Aktas et al., 2009; Armstrong et al., 2011; Baccelli et al., 2013; Hou et al., 2013; Theodoropoulos et al., 2010). Studies attempting to investigate the EMT status of CTCs have reported high expression levels of mesenchymal markers such as TWIST, AKT2, PIK3α, N-cadherin and vimentin (Aktas et al., 2009; Armstrong et al., 2011; Kallergi et al., 2011; Lecharpentier et al., 2011). While the direct clinical relevance of such markers has yet to be established, they are of significant research interest for better characterizing CTCs and understanding their roles in cancer metastasis.

Immunophenotyping of CTCs may be applied to the clinic in ways that could more directly influence therapy selection. In conjunction with other methods, human epidermal growth factor receptor 2 (HER2) expression status of breast cancer CTCs was determined through IF (Ignatiadis et al., 2011; Pestrin et al., 2009; Riethdorf et al., 2010). In prostate cancer, CTCs have been assayed for epidermal growth factor receptor (EGFR) expression (Shaffer et al., 2007), as well as androgen receptor (AR) status (Lazar et al., 2012). A functional EPISPOT assay was developed to distinguish apoptotic versus viable CTCs based on IF detection of proteins secreted by CTCs after short-term culturing (Alix-Panabières et al., 2005). In preclinical studies of M1 stage breast cancer patients, this technology shows that the secretion of CK-19 is associated with worse outcomes (Markou et al., 2011). In M1 stage prostate patients, a large fraction of CTCs secrete fibroblast growth factor-2, a stem cell growth factor (Alix-Panabieres et al., 2007).

Since CTCs are rare and heterogeneous, it is desirable to multiplex marker analysis for comprehensive single cell characterization. Currently, multiplexing is severely limited due to fluorescence spectral overlap. This is exacerbated by the fact that at least three channels are required for definitive CTC detection (i.e. CK, DAPI, and CD45). The implementation of quantum dots or other advanced imaging technologies will improve capabilities in the future. The subjectivity of IF detection must also be addressed through a greater emphasis on quantitative imaging and process automation.

3.2. Fluorescence In Situ Hybridization

Somatic alterations to gene copy numbers are a hallmark of many cancers, and can be an important prognostic marker. They can further provide valuable predictive information on therapeutic efficacy. Meng et al employed a multicolor fluorescence in-situ hybridization

(FISH) assay on CTCs isolated from breast cancer patients to determine the amplification status of the *HER2* oncogene (Meng et al., 2004). They reported some level of non-concordance between HER2 status determined by the analysis of primary tumors and with their novel CTC assay. Other studies have confirmed this observation with HER2 assays based on IF (Pestrin et al., 2009) and RT-PCR (Fehm et al., 2007). This suggests that a subset of patients with initially HER2-negative disease may develop HER2-positive CTCs over the course of progression. CTC analysis provides a minimally-invasive avenue for repeated screening for the development of genomic aberrations. It is interesting to note that CTC *HER2* amplification screening has been successfully demonstrated in even primary breast cancer patients (Fehm et al., 2009; Wulfing et al., 2006). In castration-resistant prostate cancer, multicolor FISH has shown potential as a surrogate for status determination of *AR* and *MYC* status in CTCs after immunomagnetic enrichment with CellSearch® (Leversha et al., 2009; Shaffer et al., 2007).

Oncogenic translocation is an important pathogenic mechanism for some cancers. *TMPRSS2-ERG* translocation occurs in 30-70% of therapy-naïve prostate cancer patients (Tomlins et al., 2005). Using FISH analysis on CTCs, it has been shown that *TMPRSS2-ERG* fusion can be identified in CTCs of prostate cancer patients (Attard et al., 2009; Mao et al., 2008; Stott et al., 2010b). Further, there appears to be a consistency in *TMPRSS2-ERG* translocation between therapy-naïve prostate cancer from core biopsy specimens and from CTCs at the time of castration-resistance from the same patients (Attard et al., 2009). In a recent study using FISH, it has been shown that *ALK*-rearrangement can be identified in non-small cell lung cancer patients harboring *ALK*-translocations (Pailler et al., 2013).

There is, therefore, much promise through CTC analysis for minimally-invasive tumor profiling and improved prognosis related to metastatic disease. However, the low sensitivity of current enrichment technologies remains a significant hurdle to routine clinical application. In addition, the reliability and clinical relevance of these FISH-based assays must first be thoroughly validated.

3.3 Oncogene mutation detection and genomic analysis

One of the key attractions for isolating CTCs is to enable the detection of cancer gene mutations and genomic aberrations in these metastatic tumor cells that are associated with the worst prognosis for survival, reflect drivers of active disease, and predict drug sensitivity and resistance. Using CTCs isolated with microchips, it has been shown that activating *EGFR* mutations can be effectively detected in patients with non-small cell lung cancer using an allele-specific PCR amplification technique (Maheswaran et al., 2008). Further, the *EGFR* T790M mutation that confers drug resistance can also be detected from patients who have received EGFR kinase inhibitor treatment. While allele-specific PCR is a fast and cost-effective approach to determine specific mutations, prior knowledge of the mutations are necessary for the design of amplicon primer sets.

For genes with a broader distribution of pathogenic mutations, targeted regions can be amplified from genomic DNA or cDNA of CTCs and sequenced. Mutations in multiple genes can also be examined following whole genome amplification and mutation-specific PCR in which mutations in *KRAS* and *PIK3CA* have been detected from CTCs of patients with colorectal cancer (Gasch et al., 2013). It has also been reported in castration-resistant prostate cancer that silent mutations, and small deletions and insertions in the *AR* gene can be identified from CTCs and then confirmed with biopsy or autopsy specimens, some of which were associated with resistance to androgen-directed therapies (Jiang et al., 2010).

With the ability to perform single-cell whole genome amplification, it has been shown that one can perform next-generation exome sequencing with CTCs to uncover cancer-specific

mutations, both pathogenic and passenger, which are present at very different levels of prevalence in primary and metastatic colon cancer (Heitzer et al., 2013). Once amplified, the DNA from a single CTC can also be used for array-comparative genomic hybridization analysis to uncover genomic aberrations, such as large deletions and insertions that cannot be detected with exome DNA sequence analysis (Heitzer et al., 2013). Although single-cell DNA sequencing is tedious, such studies confirm the feasibility of applying CTCs to discovery research in clinical oncology studies. Technologies for cancer gene exome analysis such as the Ion Torrent AmpliSeq™ Cancer Panels (Life Technologies, Carlsbad, CA) and the TruSeq® Amplicon-Cancer Panel (Illumina, San Diego, CA) would facilitate the analysis of cancer mutations in CTCs.

3.4 Gene expression analysis of CTCs

While the analysis of mRNA from CTCs presents significant technical challenges due to the molecule's labile nature, it is desirable due to: (1) specificity to amplicons that are highly selectively expressed in tumors but not in white blood cells; (2) the ability to detect gene translocations and alternative splice variants; (3) quantitative high-throughput technologies such as multiplex digital PCR and expression arrays for cell profiling; (4) potential application to next generation sequencing analysis.

One study on mRNA analysis explains the low abundance of EpCAM expression associated with increased vimentin in basal-like breast cancer that is poorly captured with EpCAM-based CTC purification schemes (Punnoose et al., 2010). Another study further demonstrate dynamic changes in epithelial and mesenchymal composition in circulating breast cancer cells with mixed probes using RNA in situ hybridization, particularly in CTC clusters (Yu et al., 2013). It also suggests a more mesenchymal phenotype for the basal-like molecular subtype of breast cancer. Other studies have looked at oncogenic drivers with CTC analysis. *TMPRSS2-ERG* translocation is the most prevalent pathogenic event in prostate cancer, and various attempts have been made to detect this translocation in CTCs with some modest success in correlating it with primary tumors (Danila et al., 2011a; Stott et al., 2010b). Previous studies have also shown that it is possible to detect the expression of PSA from patients with castration-resistant prostate cancer and its expression is associated with worse prognosis in the patients, while the predictive value is yet to be established (Ghossein et al., 1997; Helo et al., 2009). Such a method of analysis, once matured, would allow investigators to better detect the cancer driver in individual patients and to correlate it with clinical response. Expression analysis of estrogen receptor (ER) and progesterone receptor (PR), or HER2 signature has been explored, and studies show the feasibility of such analysis (Aktas et al., 2011a; Aktas et al., 2011b; Sieuwerts et al., 2011). As biomarkers such as HER2 in breast cancer and AR in castration-resistant prostate cancer change during the course of hormone therapies, assays that can quantify their levels as well as their signaling activities would be extremely useful to correlate with drug response and to understand the development of resistance.

Various technological advances would undoubtedly facilitate such expression analysis. BioMark digital PCR (Fluidigm, South San Francisco, CA) has been used for high degree multiplexed analysis of gene expression from single CTCs after immunomagnetic enrichment (Powell et al., 2012). The expression profiles showed a high degree of heterogeneity among CTCs, but also minimal concurrence between metastatic breast cancer patients and a panel of breast cancer cell lines, which warrants further investigation and validation (Powell et al., 2012). Further analysis is also needed to cluster CTC expression with that of known subtypes of breast cancer for both validation and clinical relevance. Strati et al performed a comparison of three RT-PCR based molecular characterization techniques in breast cancer, showing a concordance level of approximately 70% (Strati et

al., 2013). Markou, Lianidou and colleagues developed a novel liquid bead array hybridization assay for CTCs based on the simultaneous detection of 6 gene targets, demonstrating feasibility in early and late stage breast cancer (Markou et al., 2011). Next generation RNA sequencing has been performed with CTCs isolated from patients with pancreatic cancer using a microfluidic chip (Yu et al., 2012). RNA sequencing results revealed the activation of WNT signaling in the CTCs, which may contribute to tumor metastasis. Taken together, while it is very attractive to analyze mRNA for gene expression and pathway profiling, its quantitative analysis requires a high quality source of mRNA that is consistently well preserved.

4. Clinical applications of CTC assays

Due to the minimally invasive nature of obtaining blood from cancer patients and its potential implications for cancer diagnosis, there is a great interest in developing methods to extract and analyze CTCs for diagnosis, prognosis, and clinical management of cancer patients. There are several potential benefits for successful CTC or blood-based diagnostics. With the ability to obtain multiple blood draws from cancer patients throughout the duration of clinical management, the analysis of CTCs may potentially provide clinicians with information on biomarkers predictive of drug sensitivity (Maheswaran et al., 2008; Miyamoto et al., 2012), with an independent surrogate biomarker of response and patient prognosis (Cristofanilli et al., 2004; Cristofanilli et al., 2005; de Bono et al., 2008; Hayes et al., 2006), with an indicator for early relapse (Pachmann et al., 2008), as well as with material for the analysis of drug resistance (Scher et al., 2009). Because of the extreme rarity of CTCs in the circulation, while their enumeration has been widely accepted as a strong and independent prognostic biomarker for several major epithelial tumors, obtaining information on other types of biomarkers, i.e., predictive biomarkers that may affect treatment decisions, have been highly elusive and challenging to implement.

4.1 CTC enumeration as a prognostic biomarker for overall survival

Veridex's CellSearch® assay is the only FDA-cleared method for CTC enumeration. It is considered the sole CTC methodology that has been analytically validated for sensitivity, accuracy, and reproducibility (Allard et al., 2004). Using the CellSearch® assay, CTC enumeration has been established in many prospective studies to be a prognostic marker for metastatic breast (Budd et al., 2006; Cristofanilli et al., 2004; Cristofanilli et al., 2005; Hayes et al., 2006), prostate (de Bono et al., 2008; Scher et al., 2009), and colon cancer (Cohen et al., 2008; Cohen et al., 2009). The CellSeach® assay has also been shown to have prognostic utility in some lung cancer patients, particularly in those with small cell lung cancer who present with higher CTC counts in the majority of cases (Hou et al., 2012; Krebs et al., 2011). While CTC enumeration with the CellSearch® assay is the most established method, it has limitations, particularly the sensitivity of detection and information that may influence treatment decisions as well as clinical outcomes for patients. For instance, the CellSearch® assay detects ≥5 CTCs per 7.5 ml blood in only about 10% of patients with metastatic non-small cell lung cancer, which is associated with worse prognosis (Krebs et al., 2011), a sensitivity which is far less than desirable to be effective in patient management. Further, while CellSearch® Profile kits can be obtained from Veridex for IF and FISH analysis of CTCs, their performance and effectiveness have not been established for clinical usage for the analysis of predictive biomarkers (www.veridex.com).

An early stage anti-EpCAM antibody-coated microchip-based technology has been shown to be much more effective in capturing CTCs with a near 100% success rate and high CTC counts in a wide range of cancers, including all of the 55 lung cancer patient samples used in one study (Nagrath et al., 2007). Subsequent work revealed that biopsy-matching mutations in EGFR can be effectively detected from the CTCs isolated from non-small cell lung cancer

patients with this anti-EpCAM antibody-coated microchip (Maheswaran et al., 2008). However, the analysis of two commercial versions of this CTC-capturing microchip in comparison with the CellSearch® method done by Genentech Inc. fails to support such microchip specific claims, both analytically and with clinical specimens (Punnoose et al., 2010). In fact, the amount of CTCs captured with microchips was comparable to that with the CellSearch® assay. While the microchip technology has promise, the isolation technology and subsequent assays need to be sufficiently mature to be used reproducibly in different diagnostic labs. Further, associations between the number of CTCs isolated by microchips and clinical endpoints need to be established.

Various other technologies, including microchips of different isolation mechanisms (Tan et al., 2009b), microfilters (Hofman et al., 2011a; Lin et al., 2010), and methods using physical and electrical properties (Gupta et al., 2012; Hou et al., 2013; Ozkumur et al., 2013) of CTCs have been invented and evaluated for CTC isolation with various success. However, they need to be vigorously validated analytically for accuracy, precision, stability, and reproducibility. Further, prognostic or other predictive biomarkers need to be established with prospective clinical studies for these technologies to be useful (Danila et al., 2011c; Parkinson et al., 2012).

The direct analysis of CTCs by assaying all nucleated cells in blood may have some advantages due to potentially significant variations between CTC isolation technologies that may result in different rates of recovery and loss of tumor cells for different types of tumors. This approach, therefore, may be less susceptible to the variations in physical and biological properties of different types of tumors and within heterogeneous CTC populations. In fact, the recent advances in high-definition cell image analysis revealed that multiple fluorescence images of very high resolution can be obtained and analyzed from millions of cells on slides to enable CTC identification (Nieva et al., 2012; Wendel et al., 2012). This assay detects ≥ 5 CTCs per ml of blood from all stages of non-small cell lung cancer patients (Wendel et al., 2012), which initially has been associated with worse overall survival (Nieva et al., 2012). The technology is suitable for detection by IF of cancer-specific proteins and by FISH for genomic aberrations in cancer cells and thus, has a potential to integrate the analysis of one additional biomarker for correlative studies in treatment trials.

While the enumeration of CTCs with various isolation and detection technologies, particularly the CellSearch® assay, may provide valuable prognostic information, its limited utilities have restricted a broader adoption in oncology practice. In a recommendation by the American Society of Clinical Oncology, it was suggested that there were no data generated to prove that the use of this CTC test would lead to a longer survival time or improved quality of life for cancer patients, and additional studies are necessary to determine the utility of CTCs (Harris et al., 2007). The identification and analysis of biomarkers predictive of response to therapeutic agents are likely needed for such advancement and, indeed, are being intensely investigated.

4.2 CTC enumeration as an indicator of response

Strong associations between reduced CTC counts and progression-free or overall survival have also been established, in which the conversion of high to low CTC counts for patients on therapies indicates good prognosis for breast (Cristofanilli et al., 2004; Cristofanilli et al., 2005), prostate (Danila et al., 2007; de Bono et al., 2008), and colon cancer (Cohen et al., 2008). In contrast, patients whose low CTC counts at the baseline convert to high CTC counts during treatment do significantly worse. In metastatic breast cancer, CTC analysis has been evaluated as a response marker for chemo- and hormone-therapies, in which reduced CTC counts at weeks 3-5 have been correlated with radiographic response (Liu et al., 2009). It was further shown that the patients with <5 CTCs at weeks 3-5 following

treatment have much improved progression-free survival, whereas the CTC count at the baseline is not informative (Liu et al., 2009). Thus, CTC enumeration may be suitable as an adjunct to the standard methods for monitoring disease status of certain cancer patients during therapy.

CTC analysis has been incorporated into phase I and II clinical trials for the development of novel targeted therapies. In the development of abiraterone for castration-resistant prostate cancer, the analysis of CTCs was performed in two phase II studies. Between 60% and 70% of castration-resistant prostate cancer patients enrolled had unfavorable initial CTC counts (CTCs ≥ 5) of which nearly half converted to favorable counts following abiraterone treatment (Danila et al., 2010; Reid et al., 2010), providing evidence for drug activity in the patients. Subsequently, a phase III study showed that abiraterone is effective in improving overall survival for castration-resistant prostate cancer patients (de Bono et al., 2011). When performed properly, CTC enumeration can be a good response biomarker for anti-cancer therapies and could certainly have a role in the clinical development of novel therapeutics. Further, with CTCs as the only potential source of tumor material from the patients in some studies, the added ability of reliably obtaining information on cancer genomics and other molecular markers may facilitate the clinical identification and validation of predictive biomarkers for new therapeutic agents.

4.3 Exploratory studies on predictive biomarkers with CTCs

The arguments for more-extensive molecular analysis of CTCs are very compelling. Primary or metastatic cancer samples may not be available or readily obtainable. In addition, CTCs may be a source for longitudinal molecular analysis of the tumor to provide necessary molecular information during clinical management. Also, CTCs may facilitate response monitoring, residual disease assessment, and early relapse detection. Further, they may serve as a means to address issues such as inter-tumor and intra-tumor heterogeneity. While challenging, there has been consistent interest in the analysis of molecular markers of CTCs in various clinical studies (Danila et al., 2011b; Devriese et al., 2011). As the development of targeted cancer therapies will likely need companion diagnostics for the selection of appropriate patients, CTC isolation may have an edge in providing continuous access to tumor specimens without a biopsy procedure. The utility of CTCs is particularly recognizable in cases where relapsed tumors are very different from the original primary ones and when the relapsed tumors are very difficult to obtain, such as in castration-resistant prostate cancer. Early exploratory studies have been performed with CTCs for detecting specific predictive biomarkers in various cancer, including breast, prostate, colon, and lung cancer.

In the case of breast cancer, ER, PR, and HER2 status primarily inform treatment selection. There is a body of evidence that hormone receptors and HER2 status can change during relapse or disease progression (Liedtke et al., 2009; Meng et al., 2004; Simmons et al., 2009). However, some early analyses of molecular markers on CTCs presented some technical challenges. In one study with 213 preoperative breast cancer patients, CTCs were detected in 22% of the patients with the CellSearch® assay, and HER2 over-expression was detected by IF in 14 of 58 CTC-positive patients. Thus, the percentages of both CTCs and HER2-positive cells were too low to be effective (Riethdorf et al., 2010). Also, the correlation between HER2 status in primary tumors and CTCs appears to be relatively weak (Riethdorf et al., 2010), and the assay may require further validation. In another study of 254 metastatic breast cancer patients, HER2 status was analyzed with both HER2 IF with the CellSearch® assay and HER2 mRNA with RT-PCR from AdnaTest BreastCancer™. While both assays were able to detect HER2-positive CTCs in 40-50% of patients, the correlation between the assays was weak (Fehm et al., 2010). Similar results with low concordance rates were also observed with ER and PR in CTCs and primary tumors (Aktas et al., 2011b). It

was concluded that a gold standard for HER2 evaluation in CTCs is required to implement HER2 status as a stratification marker of HER2 targeted therapy in patients where repeated biopsies are not feasible (Fehm et al., 2010).

There are enormous interests in the analysis of CTCs for castration-resistant prostate cancer. The cancer generally occurs a few years after the removal of the primary cancer and tends to metastasize to bone, making them generally inaccessible to biopsy. There are further extensive changes in the tumors during the development of castration-resistance, with half of them having amplifications or mutations of the *AR* gene (Grasso et al., 2012). The development of second generation anti-androgen therapies and the absence of any companion diagnostics (Rathkopf and Scher, 2013) leave castration-resistant prostate cancer as the only remaining major cancer without methods of identifying appropriate patients for the available targeted therapies (Scher et al., 2013). While CTC enumeration has been extensively evaluated as a potential efficacy (surrogate) biomarker of survival for AR-targeted therapies, much effort was made to perform molecular profiling of CTCs, including the analysis of *AR* status, *TMPRSS2-ERG* translocation, *PTEN* deletion and *MYC* amplification with FISH-based analysis (Attard et al., 2009; Leversha et al., 2009). A study of the *TMPRSS2-ERG* fusion gene in CTCs suggests that the presence of the translocation may indicate susceptibility to the CYP17 inhibitor, abiraterone acetate (Attard et al., 2009). Using CTCs isolated with the CellSearch® instrument, there are some examples of detecting PSA expression and *AR* mutation with RT-PCR-based approaches (Helo et al., 2009; Jiang et al., 2010). Using herringbone-chip based technology, CTCs isolated from prostate cancer patients have been evaluated for *TMPRSS2-ERG* and AR signaling with IF and RT-PCR (Miyamoto et al., 2012; Stott et al., 2010a; Stott et al., 2010b) and recently, for the exploration of single cell RNA expression analysis (Ozkumur et al., 2013). While some of the technologies and exploratory studies show great promise, none of them have been clinically validated, and thus, significant advances in analytic validation and clinical qualification are required for their use in prostate cancer (Danila et al., 2011c; Scher et al., 2013).

In metastatic colorectal cancer, the final major cancer with FDA-cleared use of CellSearch, patients with ≥3 CTCs per 7.5 ml blood have significantly worse overall survival (Cohen et al., 2008). Efforts were made to explore biomarkers from CTCs in patients who had undergone curative surgery. Using total blood, mRNA and cDNA were generated and analyzed. A positive expression signature of *CEA/CK/CD133* was identified in a training set and then validated in a second validation set of patients to have worse prognosis (Iinuma et al., 2011). Successful attempts were reported in analyzing biomarkers that may predict drug sensitivity by detecting mutations in *KRAS* and *PIK3CA* from CTCs isolated with CellSearch® followed by micromanipulation (Gasch et al., 2013; Heitzer et al., 2013). Further, proof-of-principle single-cell whole genome analysis with DNA array-comparative genomic hybridization and exome sequencing have been performed with CTCs isolated with the CellSearch® instrument (Heitzer et al., 2013). Deep-sequencing analysis reveals that the method is capable of detecting mutations in *APC*, *KRAS*, and *PIK3CA* from multiple CTCs, which are also present in both the primary and metastatic tumors of the same patient (Heitzer et al., 2013). Such whole genome analysis from CTCs may have the potential to uncover biomarkers predictive of sensitivity or resistance when tumor tissues are not available.

Lung cancer is the only major cancer without an FDA-cleared CTC application. A recent study shows that in the case of non-small cell lung cancer, the most prevalent lung cancer type, only about 20% of stage III and IV patients have ≥ 2 CTCs per 7.5 ml of blood (Krebs et al., 2011). Only about 10% of these patients have ≥ 5 CTCs, which is a worse prognostic marker for overall survival. In contrast, the detection sensitivity for small cell lung cancer is

far better at 85%, and a high CTC count is a significantly worse prognostic biomarker (Hou et al., 2012). The CTCs isolated by CTC-chip have been used for EGFR mutation detection with mutation-specific PCR analysis (Maheswaran et al., 2008). While such oncogene mutations can also be detected by methods utilizing plasma tumor DNA (ptDNA), the isolation of CTCs has certain advantages in detecting gene rearrangements, cancer-specific expression, and more-extensive whole genome analysis for discovery research. For the clinical application of CTC-based assays, a recent study shows that by using the ISET filtration method for CTC enrichment, followed by in-situ hybridization, *ELM4-ALK* translocation can be detected in all 18 *ALK*-rearrangement positive non-small cell lung cancer patients, but not in the *ALK* rearrangement-negative patients (Pailler et al., 2013). If confirmed, such a method may provide a non-invasive method for the analysis of predictive biomarkers for lung cancer.

Various cell surface marker-independent technologies have the potential to isolate live CTCs, including a microfluidic chip (Tan et al., 2009b), dielectrophoretic field flow fractionation (Gupta et al., 2012), and Dean flow fractionation (Hou et al., 2013). Such methods may allow the isolation and *ex vivo* expansion of the isolated tumor cells from circulating blood for genomic analysis as well as molecular pharmacology studies. Although difficult, a recent report demonstrates the possibility of establishing rare mouse xenografts with cells from breast cancer patients who have very high CTC counts that help identify metastasis-initiating cells (Baccelli et al., 2013). It is also worth noting a recent publication in which a medium containing ROCK kinase inhibitor and feeder cells was capable of reprograming epithelial cells to enable long-term *ex vivo* culturing and expansion of a wide range of normal and tumor cells (Liu et al., 2012). Such a method could be used in conjunction with CTC isolation technology to evaluate the potential to establish CTCs *ex vivo*. The ability to grow tumor cells isolated from blood offers a very attractive option for both cancer research and patient care.

4.4 Alternative technology to CTCs, ptDNA and its analysis

As an alternative to CTCs, ptDNA has been intensively investigated to determine its feasibility as a form of ‘liquid biopsy’ for the analysis of cancer biomarkers, including particular mutations in genes that are predictive of drug sensitivity or resistance. Very significant progress has been made in this area that may represent an alternative approach to CTC-based analysis. Similar to CTC enumeration that is prognostic for cancer patients, the relative quantification of ptDNA can be determined by analyzing plasma DNA using an emulsion bead-based single-molecular amplification that is capable of determining the relative abundance of tumor DNA in plasma. Detectable ptDNA has been shown to be a better predictive marker for recurrence-free survival when compared with that of plasma carcinoembryonic antigen for colorectal cancer patients (Diehl et al., 2008). Moreover, the analysis of ptDNA from metastatic breast cancer patients has been shown to be a more sensitive biomarker with better correlation to tumor burden when compared with circulating tumor antigen CA 15-3 (Dawson et al., 2013). By using targeted deep DNA sequencing analysis, mutation in selective oncogenes and tumor suppressors can be identified, and their allele frequencies determined (Forshew et al., 2012). *KRAS* mutation is predictive of non-responsiveness to anti-EGFR therapies. Mutations in *KRAS* have been seen to emerge in ptDNA in patients that have been treated with the EGFR antibodies, cetuximab and panitumumab, indicating that developing *KRAS* mutations is a mechanism to escape EGFR blockage (Diaz et al., 2012; Misale et al., 2012). Similarly, mutations in a large number of genes can be identified with increased allele frequency in ptDNA from cancer patients who progressed on a number of targeted or cytotoxic chemotherapies using whole genome exon analysis (Murtaza et al., 2013). It is very likely that the analysis of ptDNA will have value in monitoring tumor burden and in detecting gene mutations for clinical research and for

cancer patient management. Recent advances in digital PCR technologies such as Biomark PCR from Fluidigm and Droplet PCR from BioRad will simplify the assays and speed up the adoption in the cancer research community.

5. Conclusions and future prospects

The CellSearch® Circulating Tumor Cell test is the only FDA-cleared *in vitro* CTC diagnostic in which a positive enumeration is associated with decreased progression-free survival and overall survival for metastatic breast, prostate and colon cancer patients. It is currently the gold standard method for CTC enumeration and the only one that has been both analytically and clinically validated. This test has also been shown to be useful in monitoring these patients during clinical management. However, the CellSearch® test has limitations. It has limited sensitivity and is not applicable to all types of cancer. Assays with improved sensitivity could not only be applicable to other types of cancers, but also may detect cancers at early stages when patients are more likely to benefit from therapies. Also, while extensive work has been performed with both IF and FISH to examine biomarkers that may predict drug sensitivity, these predictive biomarker studies are still at the research and development stage. Further, the CellSearch® tests have not yet been shown to lead to improved survival or quality of life.

CTCs have the promise of serving as liquid biopsies for tumors with the potential for generating information on biomarkers predictive of response and resistance. CTC isolation and analysis is one of the most active areas for translational cancer research. Various methods have been shown to be able to isolate more CTCs from a higher percentage of cancer patients. Moreover, many assays on the analysis of potentially predictive biomarkers of therapies have been explored, including IF, FISH, RT-PCR, cancer gene mutation detection, and genomic analysis. Some of them have shown success in early clinical exploratory studies. However, for them to be used for cancer management, assays and technologies need to be better validated analytically, and methods need to be standardized and reproduced by others. Furthermore, commercial success of a CTC technology requires a robust technology that is cost effective with a fast turn-around time. The eventual integration of CTC technology into clinical patient care requires the demonstration of specific utilities in treatment decisions, such as the analysis of biomarkers predictive of response or the development of CTC-based companion diagnostics for new targeted therapies.

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Abbreviations

AR	androgen receptor
CK	cytokeratin
CTC	circulating tumor cell
DEP	dielectrophoresis
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition

EpCAM	epithelial cell adhesion molecule
ER	estrogen receptor
FDA	US Food and Drug Administration
FISH	fluorescence in situ hybridization
HER2	human epidermal growth factor receptor 2
IF	immunofluorescence
ISET	isolation by size of epithelial tumor cells technique
PR	progesterone receptor
PSA	prostate specific antigen
PSMA	prostate-specific membrane antigen
ptDNA	plasma tumor DNA
RT-PCR	reverse transcription polymerase chain reaction

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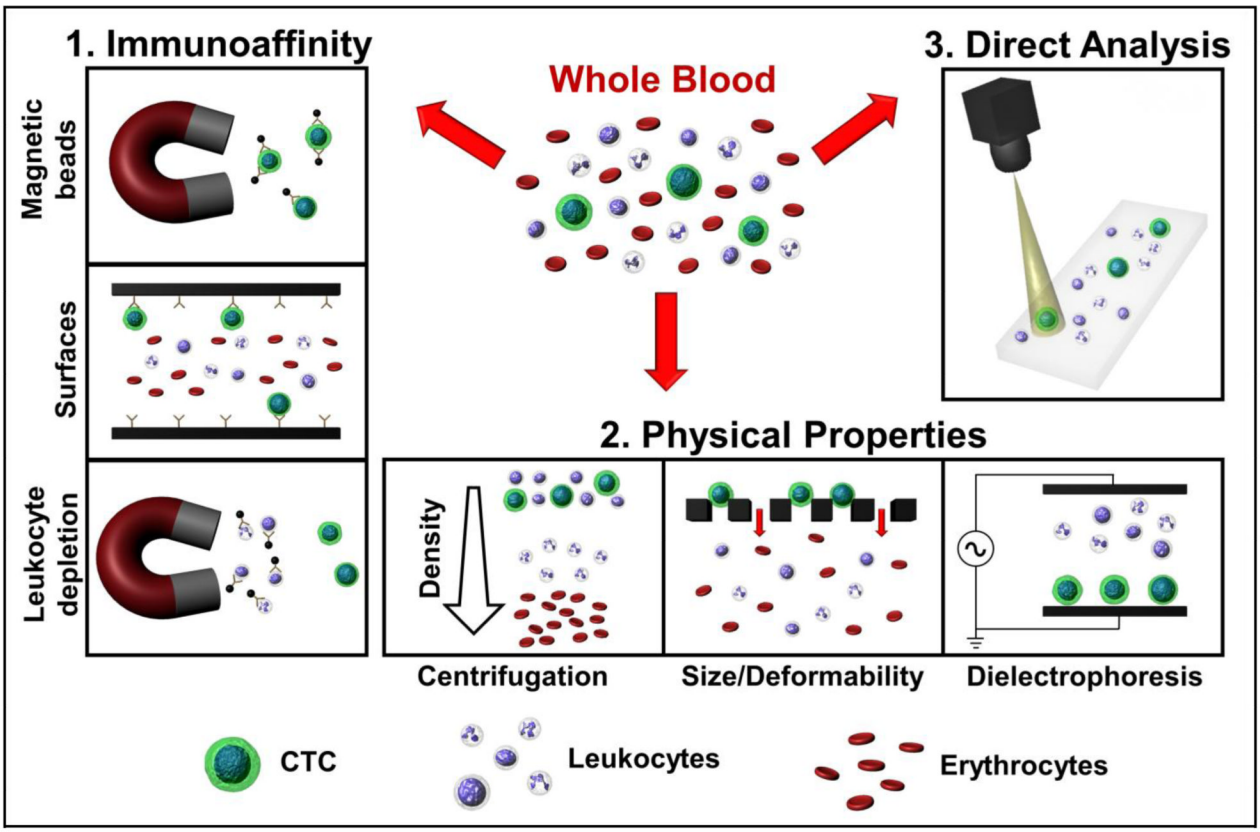

Fig. 1.

Approaches for CTC isolation from whole blood. 1: Immunoaffinity based techniques target specific markers to selectively enrich CTCs or deplete leukocytes. 2: Physical properties may be exploited to separate CTCs from blood cells based on differences in density, size, deformability and electrical properties. 3: Direct analysis is achieved by high throughput assaying of all cells in blood after erythrocyte lysis.

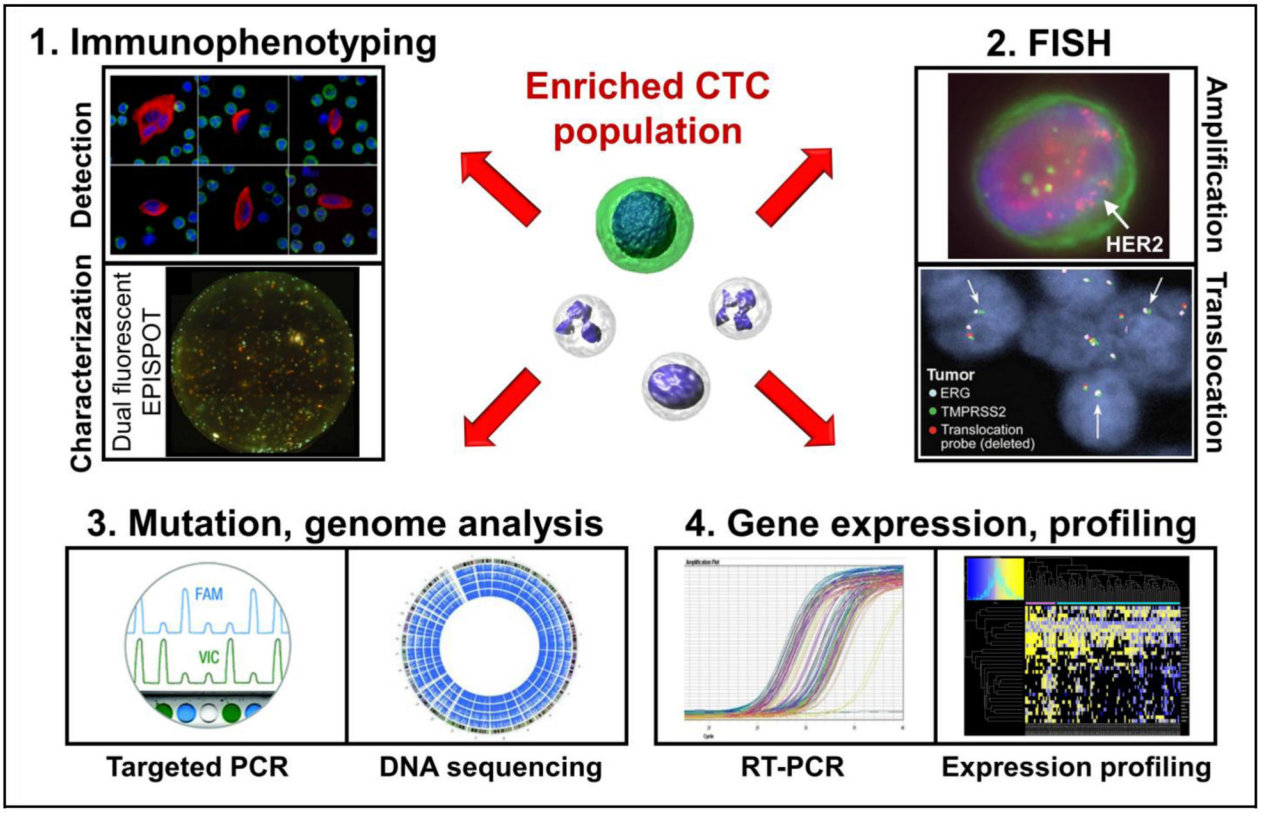

Fig. 2.

Analytic tools for analysis of CTCs after isolation. 1: Immunophenotyping employs antibodies to label proteins for CTC detection and characterization. 2: Fluorescence in situ hybridization detects aberrant amplification and translocation of specific genes. 3: PCR and DNA sequencing techniques identify oncogenic mutations and alterations to the genome. 4: RT-PCR and RNA based profiling characterize CTC gene expression.

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Detection: IOP Publishing (Marrinucci et al., 2012)

Characterization: BioMed Central (Alix-Panabieres et al., 2009)

Amplification: Public Library of Science (Punnoose et al., 2010)

Translocation: The American Association for the Advancement of Science (Stott et al., 2010b)

DNA sequencing: John Wiley and Sons (Zhao et al., 2013)

Expression profiling: Public Library of Science (Powell et al., 2012)

Table 1
Summary of technologies for CTC isolation

Isolation method	Cell line	Capture efficiency	Enrichment / (Purity)	Throughput*	Sample volume	Clinical detection	Reference
1. Immunoaffinity:							
anti-EpCAM-coated beads and magnetic sweeper	MCF7	62 ± 7%	10^8 / (51 ± 18%)	9 mL/hr	9 mL	IF: 100% (17/17) of metastatic breast cancer patients	Talasaz et al., 2009
anti-EpCAM-coated beads and magnetic sweeper	-	-	− / −	9 mL/hr	9 mL	RT-PCR: 70% (35/50) ofprimary and metastatic breast cancer patients	Powell et al., 2012
anti-EpCAM-coated beads and magnetic sifter	H-1650	91.4%	− / (17.7 ± 9.3%)	10 mL/hr	0.9 - 3.3 mL	IF: 100% (6/6) of lung cancer patients	Earhart et al., 2013
AdnaTest® BreastCancer	-	-	− / −	-	10 mL	RT-PCR: 52% (22/42) ofmetastatic breast cancer patients	Tewes et al., 2009
AdnaTest® BreastCancer	-	-	− / −	-	10 mL	RT-PCR: 19% (16/86) ofpre-surgery and 27% (19/70) of post-chemotherapy ovarian cancer patients	Aktas et al., 2011a
AdnaTest® BreastCancer	-	-	− / −	-	10 mL	RT-PCR: 19% (97/502) of primary breast cancer patients	Kasimir-Bauer et al., 2012
anti-EpCAM-coated microposts	NCI-H1650	> 60%	**10^6 / (> 47%)	1 - 2 mL/hr	0.9 - 5.1 mL	IF: 99% (122/123) of metastatic cancer and early prostate cancer patients	Nagrath et al., 2007
anti-PSMA-coated microposts	LNCaP	85 ± 5%	10^9 / (68 ± 6%)	1 mL/hr	1 mL	IF: 90% (18/20) of prostate cancer patients	Gleghorn et al., 2010
anti-EpCAM-coated herringone chip	PC3	up to 91.8 ± 5.2%	− / (14.0 ± 0.1%)	1.5 - 2.5 mL/ hr	~4 mL	IF: 93% (14/15) of metastatic prostate cancer patients	Stott et al., 2010a
anti-EpCAM or leukocyte depletion microchip	Various	up to 98.6 ± 4.3%	**10^3.5 / (0.02 - 43%)	8 mL/hr	6 - 12 mL	IF: 88% (37/42) of metastatic cancer patients	Ozkumur et al., 2013
anti-EpCAM-coated silicon nanopillar array	MCF7	> 95%	− / −	1 mL/hr	1 mL	IF: 77% (20/26) of prostate cancer patients	Wang et al., 2011
selectin and anti-EpCAM or anti-PSMA in microtubes	KG1a	~50%	− / (66.0 ± 3.9%)	4.8 mL/hr	7.5 mL	IF: 100% (14/14) of metastatic cancer patient samples	Hughes et al., 2012
in vivo sampling with anti-EpCAM coated needle	SK-Br-3	-	− / −	3 - 6 L/hr	1.5 - 3 L	IF: 92% (22/24) of breast and lung cancer patients	Saucedo-Zeni et al., 2012

Isolation method	Cell line	Capture efficiency	Enrichment / (Purity)	Throughput*	Sample volume	Clinical detection	Reference
anti-CD45 leukocyte depletion	MCF-7	46%	~1.5 × 10^5 / −	-	-	-	Lara et al., 2004
2. Physical properties:							
Ficoll-Paque® centrifugation	HT 29	-	− / −	-	10 mL	RT-PCR: 41% (24/58) of colorectal cancer patients	Weitz et al., 1998
Ficoll-Paque® centrifugation	HT-29	84%	**3.8 / −	-	-	-	Rosenberg et al., 2002
OncoQuick® centrifugation	HT-29	87%	**632 / −	-	10 - 30 mL	RT-PCR: 30% (11/37) of gastrointestinal carcinoma patients	Rosenberg et al., 2002
OncoQuick® centrifugation	MCF7	70.6%	− / −	-	20 mL	IF: 24% (30/123) of primary and metastatic breast cancer patients	Muller et al., 2005
OncoQuick® centrifugation	-	-	− / −	-	15 mL	IF: 23% (14/61) of metastatic carcinoma patients	Balic et al., 2005
Diagnostic leukapheresis and CellSearch®	SK-BR-3	-	− / −	-	~ 4.5 L	IF: 72% (21/29) of carcinoma patient samples	Fischer et al., 2013
round pore track-etched microfilter	-	-	− / −	-	6 mL	Cytomorphology: 52% (23/44) of primary liver cancer patients	Vona et al., 2004
round pore track-etched microfilter	-	-	− / −	-	7.5 mL	Cytomorphology: 95% (57/60) of metastatic cancer patients	Farace et al., 2011
round pore parylene microfilter	LNCaP	89.0 ± 9.5%	>10^6 / −	-	-	-	Zheng et al., 2007
round pore parylene microfilter	Various	92 ± 14%	10^7/ −	225 mL/hr	7.5 mL	IF: 89% (51/57) of metastatic cancer patients	Lin et al., 2010
electroformed nickel microcavity array	Various	68 - 100%	− / −	12 mL/hr	3 - 7.5 mL	IF: 88% (37/42) of metastatic lung cancer patients	Hosokawa et al., 2013
3D parylene microfilter	MCF-7	86.5 ± 5.3%	**10^3/ −	12 - 20 mL/hr	-	-	Zheng et al., 2011
slot pore parylene microfilter	PC3	~70%	**1.5 × 10^3 / −	12 mL / hr	1 mL	Telomerase assay: 46% (6/13) of metastatic prostate cancer patients	Xu et al., 2010
crescent-shaped microfuidic traps	Various	> 80%	− / (> 80%)	0.7 mL/hr	-	-	Tan et al., 2009
crescent-shaped microfuidic traps	-	-	− / −	-	1 - 3 mL	IF: 100% (5/5) ofmetastatic lung cancer patients	Tan et al., 2010

Isolation method	Cell line	Capture efficiency	Enrichment / (Purity)	Throughput*	Sample volume	Clinical detection	Reference
inertial microfluidic trapping reservoirs	MCF-7	~ 80%	**1.2 × 10^4 / −	1.2 mL/hr	-	-	Bhagat et al., 2011
inertial microfluidic trapping reservoirs	HeLa, MCF-7	10 - 23%	**5.5 - 7.1 / −	-	-	-	Hur et al., 2011
inertial microfluidic trapping reservoirs	MCF7	20.7%	**3.5×10^4 / (89%)	22.5 mL/hr	7.5 mL	IF: 100% (12/12) of breast and lung cancer patients	Sollier et al., 2013
inertial microfluidic spiral channel	Various	> 85%	**~10^3 / (~10%)	3 mL/hr	~ 6 mL	IF: 100% (20/20) of metastatic lung cancer patients	Hou et al., 2013
dielectrophoretic flow separation	SKOV3, MDA-MB-231	71.2 - 75.4%	**~10^2 / −	7.5 mL/hr	-	-	Gupta et al., 2013
3. Direct analysis:							
fiber-optic array scanning of nucleated cells	SKBR3	~ 100%	− / −	-	-	IF: 68% (46/68) of prostate, breast and pancreatic cancer patients	Marrinucci et al., 2012
fiber-optic array scanning of nucleated cells	-	-	− / −	-	-	IF: 73% (57/78) of early and late stage lung cancer patients	Wendel et al., 2012
fiber-optic array scanning of nucleated cells	-	-	− / −	-	-	IF: 68% (45/66) of samples from 28 lung cancer patients	Nieva et al., 2012
Hall-effect sensing after antibody magnetic labeling	MDA-MB-453	-	− / −	3.75 mL/hr	7.5 mL	Hall-effect sensing: 100% (20/20) of ovarian cancer patients	Issadore et al., 2012