Circulating Tumor Cells: Liquid Biopsy of Cancer
Catherine Alix-Panabières1–2,3 and Klaus Pantel4*

BACKGROUND: The detection and molecular characterization of circulating tumor cells (CTCs) are one of the most active areas of translational cancer research, with >400 clinical studies having included CTCs as a biomarker. The aims of research on CTCs include (a) estimation of the risk for metastatic relapse or metastatic progression (prognostic information), (b) stratification and real-time monitoring of therapies, (c) identification of therapeutic targets and resistance mechanisms, and (d) understanding metastasis development in cancer patients.

CONTENT: This review focuses on the technologies used for the enrichment and detection of CTCs. We outline and discuss the current technologies that are based on exploiting the physical and biological properties of CTCs. A number of innovative technologies to improve methods for CTC detection have recently been developed, including CTC microchips, filtration devices, quantitative reverse-transcription PCR assays, and automated microscopy systems. Molecular-characterization studies have indicated, however, that CTCs are very heterogeneous, a finding that underscores the need for multiplex approaches to capture all of the relevant CTC subsets. We therefore emphasize the current challenges of increasing the yield and detection of CTCs that have undergone an epithelial–mesenchymal transition. Increasing assay analytical sensitivity may lead, however, to a decrease in analytical specificity (e.g., through the detection of circulating normal epithelial cells).

SUMMARY: A considerable number of promising CTC-detection techniques have been developed in recent years. The analytical specificity and clinical utility of these methods must be demonstrated in large prospective multicenter studies to reach the high level of evidence required for their introduction into clinical practice.

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Early during the formation and growth of a primary tumor (e.g., breast, colon, or prostate cancer), cells are shed from the primary tumor and then circulate through the bloodstream. These circulating tumor cells (CTCs)5 can be enriched and detected via different technologies that take advantage of their physical and biological properties. CTC analyses are considered a real-time “liquid biopsy” for patients with cancer.

The prognosis of patients with carcinoma, even with small primary tumors, is mainly determined by the blood-borne dissemination of tumor cells from the primary site to distant organs—such as bone marrow (BM), liver, lungs, or brain—and to the subsequent outgrowth of these cells in their new microenvironment (1). Disseminated tumor cells (DTCs) and micrometastases can remain in a dormant state for many years after complete resection of the primary tumor before they give rise to overt metastases (2). DTCs derived from such metastases can recirculate through the bloodstream and may colonize other distant organs, giving rise to secondary metastases. Interestingly, animal experiments suggest that DTCs converted into CTCs can even return to the primary tumor, a process termed “tumor self-seeding” or “cross-seeding,” and can give rise to aggressive metastatic variants (3). These CTCs thereby have the potential to contribute to the development of local relapses (3), although this provocative hypothesis requires support from studies of cancer patients.

Minimal residual disease (i.e., the presence of DTCs) is undetectable by high-resolution imaging

5 Nonstandard abbreviations: CTC, circulating tumor cell; BM, bone marrow; DTC, disseminated tumor cell; ISET, isolation by size of epithelial tumor cell; DEP, dielectrophoresis; EpCAM, epithelial cell adhesion molecule; CK, cytokeratin; HER2, human epidermal growth factor receptor 2; MUC1, mucin-1; EGFR, epidermal growth factor receptor; EMT, epithelial–mesenchymal transition; DAPI, 4′,6-diamidino-2-phenylindole; RT-PCR, reverse-transcription PCR; MET, mesenchymal–epithelial transition.
technologies; however, DTCs can now be identified in the BM, lymph nodes, or circulating blood with sensitive and specific assays (4). BM is easily accessible by needle aspiration through the iliac crest, and it has played the most prominent role thus far among the distant organs as an indicator organ for minimal residual disease (5). BM appears to be a common homing organ for DTCs derived from carcinomas of different organs; it also might be a reservoir for DTCs with the capacity to reenter other distant organs (6).

For the follow-up of patients with cancer, sequential analyses are pivotal. Because BM needle aspiration is far more invasive than sampling peripheral blood, research groups are currently evaluating the clinical utility of testing for tumor cells in the blood instead of the BM to assess prognosis and monitor systemic therapy (4).

The capability to detect CTCs in the peripheral blood of cancer patients holds great promise, and many exciting technologies have been developed in recent years. Detecting CTCs remains technically challenging, however. CTCs occur at very low concentrations, e.g., a single tumor cell in a background of millions of blood cells. Their identification and characterization require methods of extremely high analytical sensitivity and specificity, which usually consist of a combination of enrichment and detection procedures. In this review, we discuss some of the key technologies of CTC detection and emphasize the remaining challenges that must be overcome to bring CTC analyses into clinical practice.

Strategies for CTC Enrichment

Approaches to CTC enrichment include a large panel of technologies based on the different properties of CTCs that distinguish them from the surrounding normal hematopoietic cells, including physical properties (size, density, electric charges, deformability) and biological properties (cell surface protein expression, viability). The current strategies for CTC enrichment are summarized in Fig. 1.

**Physical Properties**

The advantage of physical properties is that they allow CTC separation without labeling. Methods based on physical properties include: density gradient centrifugation [Ficoll, OncoQuick (Greiner Bio-One)]; filtration through special filters [e.g., the ISET (isolation by size of epithelial tumor cells) filter or a novel 3-dimensional microfilter]; a new versatile label-free biochip that exploits the unique differences in size and deformability of cancer cells (larger and stiffer than blood cells); a photoacoustic flow cytometer; a microfluidics device that combines multiorifice flow fractionation and the dielectrophoresis (DEP) cell separation technique; and a DEP field-flow fractionation device that allows isolation of viable CTCs by their different response to DEP owing to their differences in size and membrane properties (7, 8).

**Biological Properties**

*Immunobead assays.* These assays have mainly used immunologic procedures with antibodies against either tumor-associated antigens (positive selection) or the common leukocyte antigen CD45 (negative selection). Immunomagnetic assays target an antigen by means of an antibody coupled to a magnetic bead; the antigen–antibody complex is subsequently isolated via exposure to a magnetic field. Positive selection is usually carried out with antibodies against the epithelial cell adhesion molecule (EpCAM). CTCs are subsequently detected immunocytologically with antibodies to cytokeratins (CKs), the intermediate filaments of epithelial cells (7). Among the current EpCAM-based technologies, the US Food and Drug Administration-cleared CellSearch® system (Veridex) has gained considerable attention over the past 7 years (4) and is the “gold standard” for all new CTC-detection methods. Another example is the MagSweeper, which positively enriches for CTCs expressing EpCAM and allows their subsequent molecular analysis. Through the use of a 3-dimensional nanostructured substrate—a nano-“fly paper” technology that consists of a silicon nanowire array coated with anti-EpCAM antibodies—CTCs can also be captured efficiently (7).

*Microdevices.* At present, there is a focus on the development of microfluidics devices (“chips”) that can handle very small blood volumes. The first CTC chip consisted of an array of anti-EpCAM antibody–coated microposts, and the CTC chip has been developed further into a herringbone structure. Recently, investigators in the field have seen the introduction of both a new CTC chip, called “Ephesia,” which uses columns of biofunctionalized superparamagnetic beads self-assembled in a microfluidic channel onto an array of magnetic traps, and another microfluidics system that uses high-throughput selection, enumeration, and electrokinetic manipulation of low-abundance CTCs (7). The new microfluidics chip–based micro-Hall detector can directly measure single CTCs tagged with a panel of magnetic nanoparticles in a whole-blood sample (9). Capturing CTCs lacking EpCAM expression has involved the use of cocktails of antibodies against various other epithelial cell surface antigens [human epidermal growth factor receptor 2 (HER2), mucin-1 (MUC1), epidermal growth factor receptor (EGFR), folate-binding protein receptor, TROP-2] and against...
Enrichment of CTCs from the peripheral blood of cancer patients is based on the physical or biological properties of CTCs. Physical properties include size (membrane filter devices), deformability (microfluidics system in a chip), density (Ficoll centrifugation), and electric charge (dielectrophoresis). Biological properties are based on the following: the expression of cell surface markers, including an epithelial cell adhesion molecule (EpCAM) for positive selection and CD45 for negative selection; anti-EpCAM or anti-CD45 antibodies conjugated with magnetic beads, for enriching CTCs in a magnetic field; anti-EpCAM antibodies on microposts or columns of nanobeads; anti-EpCAM antibodies conjugated to 3-μm beads to increase the size of CTCs before filtration; anti-EpCAM, anti-HER2/neu, anti-EGFR antibodies on different sizes of nanoparticles for capturing and detecting different CTCs. All of these technologies have been used with blood samples ex vivo. Recently, however, a new in vivo technology (upper right) allows the enrichment of CTCs directly in the arm vein of the patient, which enables the enrichment of CTCs from approximately 1.5 L of blood. This technology therefore might be suitable to collect larger quantities of CTCs [Saucedo-Zeni et al. (24)]. Glyco A, glycophorin A—a protein of 131 amino acid residues on the extracellular surface of red blood cells (RBC). Modified with permission [Alix-Panabières et al. (7)].
mesenchymal or stem cell antigens (c-MET, N-cadherin, CD318, and mesenchymal stem cell antigen) (10). Immediate and direct microscopical analyses of single cells without any cell manipulation are now possible through the use of a transparent microchannel. CK⁺ and CK⁻ CTCs can be detected, and the complex aneuploid CK⁻ CTCs that have been isolated from clinical samples may represent epitheliomesenchymal transition (EMT)-derived CTCs.

Size-based filtration is convenient, but its efficiency is still limited, because many leukocytes remain on the membrane and the smaller CTCs pass through it. This limitation is due to the large range of CTC sizes (4–30 μm), even in the same patient. The idea of Lin et al. was to first amplify the size of the CTCs by tagging them continuously with a large number of 3-μm microbeads conjugated with anti-EpCAM antibody by means of Taylor–Gortler vortex-mixing flows and selecting them specifically through a microfluidic filtration device (11).

Strategies for CTC Detection

After enrichment, the CTC fraction usually still contains a substantial number of leukocytes, and CTCs need to be identified at the single-cell level by a method that can distinguish tumor cells from normal blood cells.

PROTEIN-BASED STRATEGIES

The CellSearch system and many other CTC assays use the same identification step: Cells are fluorescently stained for CKs (positive marker), the common leukocyte antigen CD45 (negative marker), and a nuclear dye (4′,6-diamidino-2-phenylindole, or DAPI). Through multicolor image analysis with a fluorescence microscope, CTCs are defined as CK⁺/CD45⁻/DAPI⁻ cells.

One key question is whether the detected CTCs are viable or apoptotic, because only viable cells should be able to contribute to metastasis formation. For the detection of only viable CTCs, the functional EPISPOT assay (for EPithelial ImmunoSPOT), which can be added to any kind of enrichment step, has been introduced for CTC detection. This technique avoids direct contact with target cells and assesses the presence of CTCs on the basis of secreted, shed, or released proteins during 24–48 h of short-term culture (12). The EPISPOT assay has been used to assess blood and BM samples from patients with breast, prostate, and colon cancers and has provided the first clinical data and demonstrations of the clinical relevance of viable CTCs detected by this assay (13, 14).

Because enrichment steps may lead to a bias in the selection of CTCs, high-speed automated digital microscopy using fiber-optic array scanning technology has been developed to detect CTCs that have been labeled by antibodies with fluorescent conjugates (15). Other slide-based automated scanning microscopes have been introduced for detecting CTCs, but the promising results still must be validated in large clinical studies (16).

mRNA-BASED STRATEGIES

Assays that target specific mRNAs focus on the detection of viable CTCs and are the most widely used alternative to immunologic assays for identifying CTCs. In breast cancer, an mRNA encoding a CK (CK19), has been the most frequently used in clinical studies (17); however, many transcripts (e.g., encoding CK18, CK19, CK20, MUC1, prostate-specific antigen, and carcinoembryonic antigen) are also produced at low concentrations in normal blood and BM cells (17, 18). Therefore, quantitative reverse-transcription PCR (RT-PCR) assays with validated cutoff values are required to overcome this problem. Moreover, gene transcription could be downregulated in CTCs, e.g., in the course of the EMT (19), a possibility that argues in favor of multimarker RT-PCR approaches. Recently, Markou et al. described a liquid bead array hybridization assay in which mRNA isolated from immunomagnetically enriched CTCs are subjected to multiplex PCR for simultaneous measurements of the expression of 6 genes in CTCs (20). A commercially available RNA-based CTC assay, the AdnaTest™ (AdnaGen), uses nonquantitative RT-PCR to identify cells that express the putative transcripts of tumor-specific genes after immunomagnetic capture of MUC1⁺/HER2⁻/EpCAM⁺ cells (21). The assay’s limitations, which derive from the fact that MUC1 is also expressed on activated T lymphocytes, should be considered (22).

Finally, an approach that uses telomerase-specific replication-selective adenovirus for detecting CTCs in breast cancer (23) has recently been detailed. This method has produced results comparable to the CellSearch system in terms of the CTC-detection rate, both in patients with metastatic breast cancer and in those with early breast cancer; however, there was no overlap between the 2 methods in the CTC-positive patients. This new technology may isolate CTCs with biological characteristics different from those found on CTCs detected with the CellSearch system. Another reason for the discrepancy might be that hematopoietic stem cells circulating in the peripheral blood also produce high telomerase concentrations, which may cause false-positive results.

Increasing the Yield of CTCs

Besides the choice of the appropriate CTC marker, the limited blood sample volumes available from patients...
with cancer may pose a serious limitation for the detection of rare events such as CTCs, a situation that occurs mostly in cases of localized cancer with no overt distant metastases, in which CTC counts are expected to be very low.

An elegant way to bypass this limitation is to target CTCs directly in vivo. This approach is now possible with the GILUPI nanodetector®. During the 30-min application of this device in a peripheral arm vein, up to 1.5 L of blood (including CTCs) pass the 2-cm functional area of the nanodetector, allowing a large number of CTCs to be bound by anti-EpCAM antibodies (24). The surface of the nanodetector can be coated with additional antibodies, and CTCs can be removed for immunocytochemical or PCR-based analyses. Clinical studies of patients with breast and lung cancer have demonstrated that the GILUPI nanodetector can isolate larger numbers of CTCs than with the CellSearch system (24). In addition, this nanodetector has very good biocompatibility, with no side effects demonstrated for any applications. This new nanodetector has been used with a limited number of patient samples and needs to be validated in multicenter clinical trials to prove its clinical relevance compared with the other current CTC technologies.

An alternative approach is the development of leukapheresis, elutriation for subsequent ex vivo CTC analyses that use flow cytometry, and real-time PCR for molecular characterization (25). Eifler et al. showed that leukapheresis collected $13.5 \times 10^4$ mononuclear cells with 87% efficiency. Although this approach can deliver large quantities of CTCs for molecular analyses, it needs to be adapted both to minimize the stress on patients and to the clinical routine if it is to be applied for future clinical use.

EMT as a Challenge for CTC Detection

The term “cell plasticity” refers to the ability of some cells, most notably stem cells, to take on the characteristics of other cells in an organism. EMT is a complex process that leads to cell dedifferentiation and increased motility via rearrangements of cellular contact junctions and eventually the loss of cell adhesion. During this transition, cells switch their epithelial phenotype—partially or fully—into a mesenchymal one. This mechanism, which naturally occurs during organogenesis and wound healing, is still underinvestigated with respect to its relevance for tumor cell dissemination, and its impact on technologies that identify CTCs in the blood is unknown. The EMT has been proposed to be frequently associated with cancer aggressiveness, and it might increase the ability of tumor cells to migrate (26). The reverse process, termed the “mesenchymal–epithelial transition” (MET), is thought to play a fundamental role after CTCs have settled down in distant organs and have started to form metastases in the new microenvironment. Thus far, little is known about the triggers that control the fine balance between the EMT and MET during the metastatic cascade (27).

A potentially important population of mesenchymal-like cancer cells present in the bloodstream of cancer patients is likely be missed with the standard detection criteria, which are based on such epithelial markers as EpCAM and the CKs. Although debate is ongoing concerning the relevance of the EMT in cancer patients (as opposed to experimental studies in model systems) (28), recent work suggests that the EMT might particularly affect tumor cells with stem cell–like properties (19). Therefore, there is great interest in investigating EMT markers in CTCs, and recent reports suggest that current assays based on epithelial antigens may miss the most aggressive CTC subpopulation (27). Thus, there is an urgent need for optimizing CTC-detection methods by including markers that are not repressed during EMT but that still allow the analysis to distinguish CTCs from the surrounding blood cells. For example, vimentin, the mesenchymal intermediate filament frequently expressed in carcinoma cells that have undergone an EMT, is also expressed in blood cells and therefore cannot be used alone as a CTC marker; vimentin needs to be combined with a molecular marker that identifies the tumor cells (29).

Circulating Epithelial Cells in Patients with Benign Diseases

The pivotal 2004 study that Cristofanilli et al. conducted in patients with breast cancer showed that circulating epithelial cells detected with the CellSearch system were rare in healthy women ($n=145$; mean (SD), 0.1 (0.2) cells per 7.5 mL whole blood) and in patients with benign breast disease ($n=200$; mean, 0.1 (0.9) cells per 7.5 mL whole blood) (30). None of the healthy control individuals had ≥2 such cells per 7.5 mL blood.

Surprisingly, positive events that met the criteria for “tumor cells” were detected in patients with benign colon diseases, both with the CellSearch system (11.3%) and with the CK19 EPISPOT assay (18.9%), whereas no positive events were detected in samples from healthy volunteers (31). Positivity was detected most frequently in patients with diverticulosis and Crohn disease, and there was no association with the development of colon cancer during the 3-year follow-up period. All positive events lacked expression of CD45, the common leukocyte antigen. These results indicate that patients with benign inflammatory colon diseases in particular can harbor viable circulating epithelial cells that can be detected with current CTC assays.
Interesting results were also found for a group of 25 men with increased serum concentrations of prostate-specific antigen who were biopsy negative for prostate cancer (32). Surprisingly, 8% of this control group had $\geq 3$ CTCs per 22.5 mL; however, the follow-up was too short to exclude the possibility that prostate cancer might be diagnosed in these men in repeat prostate biopsies.

Interestingly, results with a mouse model showed that pancreatic cells with a mesenchymal phenotype and stem cell properties circulated in the blood and seeded the liver before any primary tumor was detectable (33).

In conclusion, not only do these findings point to the need for further molecular characterization of circulating epithelial cells, they also have important implications for the use of CTC testing (31, 34).

**CTC Analysis as a Real-Time Liquid Biopsy**

A liquid biopsy may be defined as a blood test so analytically sensitive that it can detect a single tumor cell lurking among a billion normal hematopoietic cells. This new biopsy would be carried out in real time and allow the characterization of specific subpopulations of CTCs; it could revolutionize cancer detection and management (Fig. 2).

Currently, the choice of a targeted therapy for an individual patient is made after analyzing the primary tumor for the expression and/or genomic status of a specific molecular target. Making this choice is often hampered, however, by the heterogeneity and plasticity of individual tumor cells in this tissue (35). Several studies have shown that metastatic cells may have phenotypic and genotypic characteristics that are distinct from those of the bulk of the primary tumor (36). These characteristics can be explained by the facts that (a) the metastatic subclone within the primary tumor might be small and easily missed and (b) metastatic cells may gain additional genomic characteristics over time and develop independently from the primary tumor (37). Thus, direct analysis of metastatic cells could provide important additional information before patients are stratified to expensive therapies that might have considerable side effects. For example, information regarding the expression of the estrogen receptor or the HER2 oncogene on CTCs might be helpful for stratification and monitoring of endocrine therapy or therapy with trastuzumab (human anti-HER2 antibodies). Increasing numbers of reports are indicating a clear discrepancy between the status of primary tumors and CTCs from the same patients in the expression of these targets (38, 39). Moreover, CTCs from individual patients show striking heterogeneity with respect to the status of genes that express therapeutic targets such as HER2 or EGFR (38, 40, 41). The extent to which this heterogeneity may contribute to the escape of metastatic tumor cells from targeted therapy remains the subject of future clinical studies. Such analyses may also include downstream components within signal-transducing pathways that influence new targeted therapies [e.g., KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) mutations in EGFR-targeted therapies, or PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; formerly known as PI3K) mutations in HER2-targeted therapies]. Moreover, administration of trastuzumab could eliminate chemotherapy-resistant CK19 mRNA–positive CTCs, reduce the risk of disease recurrence, and prolong the disease-free survival time (42).

Therefore, implementing CTC analyses as a liquid biopsy might provide new insights into the complex mechanisms of drug resistance.

**Conclusions and Perspectives**

A number of promising CTC-detection techniques have been developed in recent years, and they continue to be improved. These new approaches must be validated in multicenter clinical studies with defined end points, such as disease-free or overall survival. In addition, we must identify the most aggressive subset of CTCs that are the metastasis-initiating cells (43). Therefore, we need to develop better strategies that are also able to isolate and identify subpopulations of tumor cells with downregulated expression of epithelial proteins. Moreover, it might become possible to identify the tissue of origin of CTCs by using expression profiling to detect organ-specific metastatic signatures in CTC cells. That would help to localize small, occult metastatic lesions and guide further diagnostic and therapeutic strategies.

Independent of the technical approach used, the key question to be addressed in clinical-intervention trials is how the assessment of CTCs will guide therapy toward more efficient elimination of metastatic cells. The ability to detect and eradicate metastatic cells at an earlier point in time clearly has the potential to decrease cancer mortality.

In addition, there is still a great interest in studying cell-free tumor DNA in parallel with CTCs (44, 45). The emergence of KRAS mutations is a mediator of acquired resistance to EGFR blockade, and these mutations—like

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6 Human genes: HER2, human epidermal growth factor receptor 2 [current HUGO-approved symbol and name: ERBB2, av-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)]; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; PIK3CA (formerly known as PI3K), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha.
CTCs can be derived from different sources (e.g., primary tumor or organs of metastasis, such as liver, lung, and BM) depending on disease stage. CTCs serve as a liquid biopsy of cancer and reveal important information on therapeutic targets and/or resistance mechanisms, which might be used in the future to stratify patients for such targeted therapies as inhibition of EGFR/HER2 or endocrine therapy and to monitor the efficacy of treatment and the development of resistance in real time. ER\(^+\), estrogen receptor positive. Modified with permission [Alix-Panabières et al. (7)].
the molecular characterization of CTCs—can be detected directly in a noninvasive manner in the serum of cancer patients. CTC analyses and cell-free DNA may explain in a synergistic way why solid tumors develop resistance to targeted therapies.

Overall, there is increasing evidence that CTCs reflect cancer progression in real time and that this information may be particularly helpful in the context of systemic therapies. In the future, CTC characterization is expected to contribute to guiding specific targeted therapies to a defined population of cancer patients within a certain therapeutic window—which is the hallmark of personalized medicine.

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**References**


43. Wicha MS, Hayes DF. Circulating tumor cells: Not all detected cells are bad and not all bad cells are detected. J Clin Oncol 2011;29:1508–11.
