

Organ-specific markers in circulating tumor cell screening: an early indicator of metastasis-capable malignancy

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Circulating tumor cells (CTCs) represent an important biological link in the spread of primary solid tumors to the metastatic disease responsible for most cancer mortality. Their detection in the peripheral blood of patients with many different carcinomas has shown that tumor-cell dissemination can proceed at an early stage of tumor development and their presence is associated with poor clinical outcomes, particularly in metastatic disease. In this article we describe how the increasingly sensitive isolation and detailed molecular characterization of CTCs has greatly improved our understanding of metastatic proliferation. We focus on how CTC detection and knowledge of the molecular architecture of these cells can serve as biomarkers to signal metastasis-capable disseminating cells and predict therapy-specific response. This has marked clinical utility for improved selection of systemic therapies to the individual needs of a cancer patient, real-time monitoring of metastatic disease treatments and the development of new targeted therapies.

Carcinomas develop from epithelial cells lining organs such as lung and colon or glandular cells and their progenitors, as in breast and prostate. Worldwide, they account for the vast majority of malignancies [1]. The metastatic spread of cancer cells in the blood and subsequent growth of secondary tumors at distant organs is the major cause of cancer mortality from solid tumors. Though carcinomas are being diagnosed at increasingly earlier stages and even with complete resection of their primary tumors, patients still have a marked risk of developing local recurrence or succumbing to metastatic relapse owing to minimal residual disease (MRD) caused by tumor cells shed from the primary carcinoma before, or during, surgery [2–4]. Histological heterogeneity of these carcinomas further complicates individual treatment options. To reduce mortality and recurrence of these cancers, improved screening, characterization of the carcinoma and ability to predict recurrence are major challenges for clinical oncologists.

As distant metastases are the major problems when treating solid tumors, the development of a specific, sensitive, noninvasive diagnostic method for the early detection and monitoring of micrometastasis should greatly improve patient prognosis. The early dispersal of tumor cells in patients with no overt metastasis usually eludes detection even by high-resolution

imaging techniques, impacting on early intervention efforts. In recent years, a plethora of sensitive and specific molecular and immunocytochemical assays have been developed that permit detection of single and small clumps of disseminated and/or circulating tumor cells (DTCs/CTCs) in the regional lymph nodes, bone marrow (BM) and peripheral blood, which appears to precede occurrence of incurable overt metastases [2,5–7]. As repeated peripheral blood sampling is more desirable for the clinical management of cancer patients than invasive BM procedures, there has been much focus on enhanced techniques for the isolation, phenotyping and genotyping of rare CTCs. This ability has led to exciting developments in investigating the systemic dissemination of CTCs in the peripheral blood, a crucial initial stage in the metastatic cascade.

This article describes the current major clinical use of CTCs as potential monitoring tools for the risk of metastasis and for treatment efficacy, highlighting studies in breast, colorectal, non-small-cell lung and prostate cancers as the most frequent solid tumors. After a brief summary of the currently available methods for the detection and molecular characterization of CTCs, we consider the clinical function of CTC analyses in cancer prognosis, therapeutic decisions and treatment monitoring. We discuss how research on CTCs is contributing to a fuller

Keywords

■ circulating tumor cell
■ metastasis ■ predictive
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■ theranostic ■ tumor
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grasp of the intricate metastatic process and the relationship with cancer stem cells. We focus on how the biological properties and molecular characterization of CTCs can help locate organ-specific markers for the early indication of malignancies with high metastatic potential and the promising role of these biomarkers in the development of targeted therapies to eliminate or manage metastatic cells before their evolution into critical overt metastases. This increased understanding of the metastatic process is crucial as 90% of cancer patients die from metastatic disease [5].

Methods for detecting CTCs

Substantial progress has been made in recent years to improve and automate the isolation and characterization of CTCs, increasing the sensitivity and specificity of CTC detection. These techniques can identify CTCs at frequencies of one per 10^6 – 10^7 nucleated blood cells [5] and three to five or more CTCs per 7.5 ml blood sample has been a typical reference for cancer prognosis based on patient survival studies. As the advantages and disadvantages of the different detection methodologies have recently been extensively reviewed ([2,5,7–10]; see in particular Table 1 in [7], and [10] for a discussion on the technical and statistical considerations for CTC analyses in the clinic), they are briefly summarized here while this article will focus on their application in the metastatic setting. The current main detection methods involve a pre-enrichment step of the CTCs followed by cytometric/immunological or molecular approaches, such as immunological assays of histogenic protein-directed monoclonal antibodies and quantitative real-time PCR (qPCR) based genetic typing of tissue-specific transcripts. Owing to the lack of tumor-specific target antigens, several of the most commonly used techniques detect CTCs indirectly by the use of antibodies against epithelial antigens, such as cytokeratins (CKs; intermediate filament proteins) (e.g., CellSearch™ [Veridex LLC, NJ, USA]), surface adhesion molecules or growth factor receptors [2,5,11]. However, these approaches suffer from a high risk of false positives (epithelial nontumor cells) and false negatives (malignant cells not expressing epithelial antigens) [5]. Moreover, the false negatives may represent the most clinically relevant CTCs as the process of the epithelial-to-mesenchymal transition (EMT) – where primary tumor cells during disease progression undergo morphogenetic changes such as downregulation of

epithelial antigens (e.g., EpCAM and CKs) to a mesenchymal-like phenotype – appears to affect key disseminating cancer cells with stem cell-like properties in particular [12]. Certain intracellular CKs are targets for particular epithelial cell types, for example, CK18 and CK20 are frequently used for CTC detection in colorectal cancer (CRC) patients and CK19 for breast cancer [13].

Successful application of molecular genetic techniques for DTC/CTC detection have principally involved reverse transcription PCR (RT-PCR) assays of epithelium-specific mRNA transcripts, such as CK18, CK19, CK20, ERBB2, Mucin 1 (MUC1), mammaglobin, Prostate-Specific Antigen (PSA) and Carcinoembryonic Antigen (CEA) [2,9]. However, RNA targeting techniques suffer from inadequate specificity owing to the lack of robust cancer-specific RNA markers. Other chief weaknesses of RT-PCR approaches include that the tumor cells cannot be morphologically identified and isolated for further analyses, low RNA stability, background levels of transcription (illegitimate transcription) in normal cells, genomic DNA and leukocyte contamination, variations in endogenous control gene expression and expression level heterogeneity of target transcripts between CTCs [2,5,14–18]. Furthermore, as during the EMT process, gene transcription can also be downregulated in CTCs/DTCs [19], a discriminatory multimarker RT-PCR panel with well-defined cutoff values (qPCR) is probably required. However, the clinical utility of qPCR assays with validated cutoff points for detection of DTCs/CTCs have been highlighted in several reports (reviewed in [2,13,20]). These approaches allow investigator-independent, quantitative and dynamic assessment of markers related to CTCs/DTCs, as compared, for instance, with the Cell Search method, which implies an investigator-dependent selection of which cells are cancer cells and, thus, a potential source of bias.

Distinct from immunocytochemistry or quantitative RT-PCR, the EPISPOT assay, based on the secretion or active release of specific marker proteins, such as cathepsin-D, MUC1 and CK19, tags viable CTCs for culturing, thus avoiding consideration of nonviable CTCs that are, therefore, not metastatic competent [21–23]. However, as this method uses epithelial targeting enrichment markers to select, for example EpCAM⁺ or CK⁺ cells, there is a lack of specificity for tumor cells transformed by EMT and the epithelial targets may also tag normal cells

that will contribute to the protein secretions assayed from the cultured cells. In addition to these methods, many others (reviewed in [7–9]) have been recently developed using advanced bioengineering technologies including microfluidic CTC chips, ultra-high-speed automated digital microscopy laser scanning cytometry (MAINTRAC [201]), size separation methods/membrane microfilter devices and Raman spectroscopy [24]. Microfluidic CTC chips containing anti-EpCAM coated microposts have captured CTCs directly from blood in almost all cancer patients independent of disease stage, although specificity of this assay needs to be fully investigated owing to frequent positive findings in healthy controls [25–27]. An automated immunomagnetic separation technology (MagSweeper® [Illumina Inc, CA, USA]) can isolate enriched populations of CTCs expressing EpCAM [28].

Enrichment methods have been developed to avoid the problems associated with targeting specific markers by selecting tumor cells out from the huge background of normal blood leukocytes based on their greater cellular size. These techniques include membrane filter devices such as Isolation by Size of Epithelial Tumor cells (ISET™ [Rarecells SAS, Paris, France]) [5,29,30] or microelectromechanical system microfilters [31], which can both isolate intact CTCs for downstream molecular applications. ISET is a powerful, easy to perform and rapid approach for enriching and isolating CTCs on polycarbonate membranes, which eliminate peripheral blood leukocytes by filtration through the calibrated pores of 8 µm. The vast majority of mature lymphocytes and neutrophils, which have a size of 8 µm and which have a compact nucleus and a tiny cytoplasm, are lost and a minority remain on the filter, sometimes trapped in the pores that are of the same size. Cancer cells, including stem cells, have a larger nucleus as their chromatin is known to be ‘open’ and the DNA is actively transcribed; their size is approximately 10–12 µm or larger; they are thus collected on the filter. Microelectromechanical system is a more complex parylene membrane microfilter device with thousands of filtering pores for cell immobilization coupled with embedded electrodes, permitting *in situ* cell lysis.

Obviating the need for repeated blood sampling, a new *in vivo* approach to CTC detection has been demonstrated in a mouse model, though not in humans as yet. Here, two-color photoacoustic flow cytometry was used to record CTCs targeted with markers for cancer

cells consisting of folate-conjugated nanotubes and magnetic urokinase plasminogen activator-conjugated nanoparticles [32]. However, this approach is likely to have insufficient specificity for identification of CTCs as folate and urokinase plasminogen activator expression markers would also be detected in hematological cells.

Importantly, validation of the clinical utility of all these newer methods by independent research groups in multicenter trials involving many cancer patients is required, particularly for robust measurements of rare CTCs at early tumor stages [10,33]. In the few studies that have directly compared isolation techniques on the same samples, results indicate an appreciable variety in detection rates [34]. Implementation of CTC measurements in clinical practice is severely hampered by this lack of standardization. There are continuing efforts to address the standardization of CTC detection protocols [35], such as the automated enrichment and immunostaining CellSearch™ system, cleared by the US FDA for CTC definition in patients with metastatic cancers of the breast, prostate and colon [33]. However, during the EMT, epithelial antigens, including EpCAM, may be downregulated, thus limiting effectiveness of EpCAM-based CTC enrichment techniques, such as CellSearch [5].

Therefore, although CTCs can currently be isolated and molecularly characterized through increasingly ingenious strategies, there remain key requirements to improve standardization criteria and the enrichment and detection of tumor cells following EMT, as noted by a recent comparison of CellSearch and two biochip platforms [36]. Thus, filtration methods, such as ISET, which allows for a ‘cell size-based’ selection of tumor cells (including those subsets of disseminating cells that lack epithelial markers) and greatly depletes peripheral blood leukocytes, show particular promise for clinical use [5,29,37].

CTCs, cancer stem cells & the metastatic process

The conventional view posits that the metastatic ability of a cancerous cell is acquired late in tumor development. However, compelling evidence to the contrary, particularly in breast cancer, suggests invasion of primary cancer cells can occur early in tumorigenesis and that CTCs may circulate months and years before metastatic development [5,38,39]. The connection of CTCs/CTCs to cancer stem cells is complex and currently under debate. A cancer stem cell marker phenotype (CD44⁺/CD24^{-/low}

or CK19⁺/Muc-1⁻) has frequently been observed in DTCs from breast cancer patients [23,40]. An expression profile in stem cells, located from the primary tumor using the CD44⁺/CD24^{-/low} markers strongly associated with metastatic relapse compared with primary breast cancer cells, reinforces the connection between breast cancer stem cells and metastasis [41]. In agreement with characteristics of cancer stem cells, DTCs/CTCs may be nonproliferating (Ki-67-negative) and chemotherapy resistant, probably because they can exist in a 'quiescent-like' state for many years [42–46].

A subset of primary tumor cells appear to undergo EMT during disease progression, involving morphogenetic changes (e.g., downregulation of EpCAM and CK) to a mesenchymal-like phenotype of enhanced motility and plasticity expediting dissemination in the blood and extravasation of cells into distant organs [5]. Implicitly, some CTCs, at least following EMT, must possess the tumor-initiating characteristics of cancer stem cells, but they must also have extra-intravasation and extravasation abilities.

To develop cell clusters (micrometastases) at the secondary organ site, these DTCs must revert to their epithelial character through the reverse mesenchymal-to-epithelial transition. Other subpopulations of CTCs exist that are not able to undergo EMT and, although dissemination to distant organs is possible, these cells cannot form metastases as they lack stem cell features (see Figure 2 in [7]). Importantly, many of frequently used CTC technologies only capture these CTCs, neglecting those with an EMT phenotype [5,7]. The presence of CTCs in the blood is also significantly correlated with metastatic relapse [47–50], suggesting that metastatic stem cells may be detectable in the blood as circulating tumor stem cells. Improved stem cell markers to CD44, such as aldehyde dehydrogenase 1 (ALDH1) [51] and BMI1 mRNA [52] will help improve stem-cell profiling of CTCs. Pertinently, the majority of CTCs from metastatic breast cancer patients expressed traits of stem cells, including ALDH1, and the EMT in a study using the AdnaTest KitTM (Zeus Scientific Inc., NJ, USA) stem cell and EMT RT-PCR assay [53], and a subpopulation of CTCs with a putative stem cell phenotype (CD44⁺/CD24⁻ or ALDH⁺/CD24⁻) were also detected in the blood of metastatic breast cancer patients using immunofluorescence microscopy [54].

As only a small proportion of primary tumor cells are probably capable of metastatic growth, the phenotype of the primary tumor and the

metastatic disease may differ. Much recent debate suggests that it is unlikely that all DTCs and CTCs harbor the necessary genetic properties to form a new metastatic tumor [2,6,20,55,56]. Equivalent genomic descriptions of breast cancer DTCs from BM do show a high degree of genetic heterogeneity [57] and genomic aberrations observed in the primary tumors were not found in the DTCs [58]. Perhaps the DTCs experienced substantial genetic changes after BM dissemination, or they may have originated from small subclones within the primary tumor that were not investigated.

Emerging data on the genetics of DTCs and CTCs have led to new insights into the metastatic process, integrating complementary concepts of the metastatic stem cell and the parallel metastatic progression models (see Figure 2 in [2]). Epithelial stem cells are the principal font of carcinogenesis under the cancer stem cell hypothesis. Tissue hierarchy is hypothesized to be preserved during malignant progression, resulting in a tumor with only a small component of cancer stem cells capable of self renewal and formation of overt metastases following dissemination. By contrast, DTCs without stem cell properties encode a limited ability to proliferate. For the metastatic stem cell model, data from breast cancer studies indicates that CTCs are released early from the primary tumor and that any resulting overt metastasis may be due to genomic changes distinct from those implicated in evolution of the primary tumor growth [2,20,38,56,59]. In other adenocarcinomas, the genetic data from DTCs revealing that metastatic cells can exist in an early-stage tumor suggest parallel metastatic progression models where a metastatic subclone already exists in the primary tumor. For example, genetic abnormalities of CTCs in early-stage patients with multifocal and heterogeneous prostate cancers match those in distinct, small focal areas of the primary tumor [60–62]. These early disseminated cells may undergo genetic progression, forming overt metastases in parallel encompassing genetically different tumor cells from those in the primary cancer. A recent intriguing whole-genome sequencing strategy showed that primary pancreatic tumors are organized in numerous, geographically distinct subclones and that metastatic cancer clones evolved within the primary tumor [63].

In summary, investigations of stem cell markers and cancer stem cells have marked implications for CTC research as, consistent with the

hypothesis of tumor stem cells, CTCs may differ in their metastatic potential so that only some have clinical relevance as metastatic precursor cells [6,23,64,65].

Current clinical relevance of CTCs

Prognosis in cancer patients & prediction of metastatic relapse

The BM appears to be a universal homing organ and reservoir of DTCs from all solid carcinoma sites, from where they can recirculate into diverse organs and, perhaps, back to the primary site [39,55]. Data, mainly from studies of breast cancer but also from colon, lung and prostate cancer, associate metastatic relapse with the presence of DTCs at primary surgery [13,39,66,67]. These observations suggest that initiator cells of overt metastases circulate among the DTCs [9]. Studies of breast and ovarian cancer patients indicate higher risks of late metastatic relapse correlate with DTCs that survive chemotherapy and hormonal therapy [42,68], and reside in the BM for several years following primary cancer surgery [46,69,70]. Owing to such results, the American Society of Oncology recommendations on tumor markers included DTCs and CTCs for breast cancer in 2007 [71]. The BM is a promising target organ for therapy and detection of DTCs could be used to monitor therapies (e.g., bisphosphonates) targeted to BM-tumor interactions [72]. A report in nonmetastatic breast cancer indicated that hormonal and radiotherapies could help prevent DTCs from reseeding the primary tumor area [73].

Minimal residual disease denotes the presence of tumor cells undetected by routine diagnostic procedures for tumor staging following surgery. The detection and monitoring of MRD by invasive BM aspiration through the iliac crest is not used in the clinical management of patients with solid tumors, although it is a standard of care for leukemia and lymphoma patients. Aspiration of BM is invasive and uncomfortable for the patient, takes time and is problematic for standardizing sample quality [6]. Moreover, biopsy procurement from primary tumors and tumor resection may stimulate cancer cell spreading [74]. Instead, as repeated peripheral blood sampling is more patient friendly than BM or tumor biopsy analyses, there is currently much effort to establish the clinical value of CTCs for prognosis, appraisal and monitoring of systemic therapy. In addition, sampling methods for CTCs are easy to repeatedly and rapidly perform, offering much greater widespread applications than for BM [67].

The prognostic relevance of CTCs in patients with early-stage cancer without overt metastasis appears to be less evident than that for DTCs in BM, although there are only a limited number of comparison studies [42,75–77] (reviewed in [2,6,9]). A quantitative RT-PCR assay for CK19 and mammaglobin mRNAs, used to analyze patients with metastatic and nonmetastatic breast cancer, showed that the detection of DTCs in BM had better prognostic ability than CTC typing [78]. These differences would be expected as blood analyses are more of a ‘real-time’ sampling of tumor cell dissemination, whereas BM may attract DTCs and aid their survival. Furthermore, the comparison of DTCs and CTCs clinical impact is dependent on the specificity of the approach used to identify tumor cells. DTCs in the BM would be more specifically detected by methods based on epithelial cell markers (e.g., CK and EpCAM), as epithelial cells are meant to be absent in the BM, while CTC identification, using RT-PCR-based techniques (some have shown promise as prognostic indicators [45,74,79–84]) or epithelial markers, is expected to be less specific and needs further validation studies [79].

Enumeration of CTCs before and after chemotherapy was demonstrated to be independently predictive of progression-free and overall survival (OS) in metastatic breast cancer [47–49]. The link between CTC detection and disease progression was, later, also observed for metastatic prostate and colon cancer [47,80,81]. Levels of CTCs appear superior or additive in predicting OS in metastatic breast cancer compared with conventional imaging methods, including computed tomograms [82,83]. Conversely, studies of patients with overt distant metastases indicate that a substantial number are negative for CTCs. One key explanation is that many of the current and frequently used detection techniques, including CellSearch, are not suited to finding CTCs with an EMT phenotype [72], which other techniques, such as ISET, capture more efficiently [5].

Use of CTCs in monitoring of systemic cancer therapies

Detection of CTCs in the blood that may signal the development of metastatic disease, provides an avenue to investigate the sequence and biology of metastasis and biomarkers of metastasis-capable malignancy [6] (FIGURE 1). Increasing knowledge of CTC biology should provide novel data to help tease out the mechanisms of metastasis [64]. In this context, there

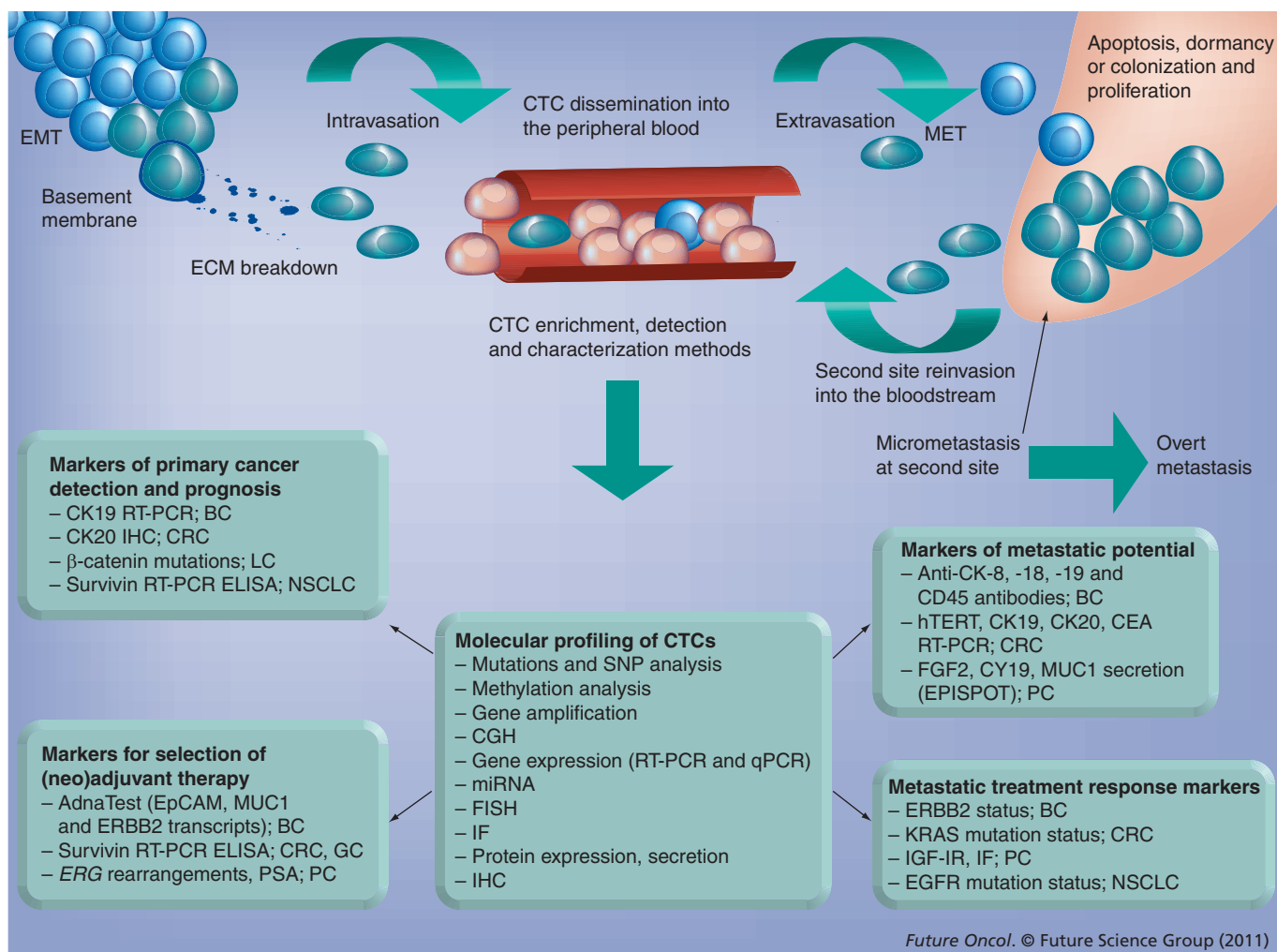


Figure 1. Schematic representation of tumor dissemination and clinical relevance of circulating tumor cell biomarkers.

A subpopulation of primary tumor epithelial cells (green) undergoes EMT and acquires invasive properties. In combination with protease stimulation, to disrupt the basement membrane and the ECM this leads to intravasation of these rare tumor cells into the bloodstream (as CTCs) where they mix with billions of normal blood cells (red). It is thought that some of these specific CTCs have cancer stem cell-like properties (as previous primary tumor stem cells and/or acquired by genetic progression in the disseminating cells) and are able to extravasate as disseminated tumor cells at distant organ sites such as the liver, bone, lung and brain. This clinically important biological subset of CTCs needs to be more efficiently detected because many of the foremost current detection techniques based on epithelial markers detect another population of CTCs (blue), which can also disseminate through the blood into distant organs, but lack stem-cell properties, are not able to undergo EMT and do not form metastases. Disseminated tumor cells need to re-express their epithelial properties via the MET to form tumor cell clusters (micrometastases). CTCs isolated from the peripheral blood can be characterized by a myriad of molecular techniques. Some examples of CTC biomarkers are shown that can be used as clinically relevant indicators at all stages of carcinogenesis and are providing insight into the biology of the metastatic process. BC: Breast cancer; CEA: Carcinoembryonic antigen; CK: Cytokeratin; CRC: Colorectal cancer; CTC: Circulating tumor cell; ECM: Extracellular matrix; EGFR: EGF receptor; EMT: Epithelial-to-mesenchymal transition; FISH: Fluorescence *in situ* hybridization; GC: Gastric cancer; hTERT: Human telomerase reverse transcriptase; IF: Immunofluorescence; IHC: Immunohistochemistry; LC: Liver cancer; MET: Mesenchymal-to-epithelial transition; MUC: Mucin; NSCLC: Non-small-cell lung cancer; PC: Prostate cancer; qPCR: Real-time PCR; RT-PCR: Reverse transcription PCR; SNP: Single-nucleotide polymorphism.

are many ongoing clinical studies to assess the huge potential of CTC analyses to better evaluate and monitor therapy as previous investigations, particularly of metastatic breast cancer have provided considerable prognostic information [6,47,49,64], seemingly superior to imaging techniques [25,82,83]. A key prospective, randomized trial to assess the clinical utility of CTC counts by the Southwest Oncology Group

[202] is examining whether women, displaying elevated CTCs at first follow-up (3 weeks) of metastatic breast cancer treated with chemotherapy, benefit from switching to an alternative chemotherapy early rather than waiting for clinical evidence of progressive disease.

Furthermore, a recent study indicates that the type of treatment may impact on the clinical relevance of CTC counts. Treatment of

metastatic breast cancer patients with bevacizumab (a monoclonal antibody that recognizes VEGF-A), in combination with first-line chemotherapy, appears to alter CTC predictive value. The authors propose that this is possibly due to impaired tumor-cell intravasation through the blood vessel endothelium [84].

Intriguingly, these recent data argue that CTCs are more metastasis competent than previously believed and could play an important role in the clinical management of nonmetastatic cancer. In primary breast cancer patients the detection, by CellSearch, of just one CTC in 7.5 ml of blood before neoadjuvant chemotherapy is a strong prognostic factor predicting OS [84,85]. In addition to breast cancer, the presence and characteristics of CTCs in 208 preoperative non-small-cell lung cancer (NSCLC) patients was recently assessed by cytological analyses after their isolation by ISET [86]. While CTCs were not detected in the control group ($n = 39$), an appreciable percentage (49%) of cases harbored CTCs, and malignant cytopathological features of the CTCs were observed in 36% of the patients. A rank of at least 50 CTCs was independently associated with shorter overall and disease-free-survival in these patients with resectable NSCLC.

In conclusion, investigators are increasingly examining the clinical utility of CTCs as they are much more readily accessible than the reservoir of DTCs in the BM. Clarification of whether BM analysis can be adequately replaced by CTC testing for the future clinical management of cancer patients requires further powerfully designed studies of larger patient cohorts (with the improved CTC detection technologies discussed previously). In addition, in other tumors where overt BM metastases are rare (such as gastrointestinal cancer), CTC examination has offered prognostic value and shows promise as an early indicator of tumor cell spread to distant organs [87,88]. A recent meta-analysis concluded that CTC detection is a significant prognostic factor for OS and recurrence risk in CRC patients [89].

Genetic characterization & molecular phenotyping of CTCs in metastatic disease

Detailed molecular analyses of CTCs, including single microdissected CTCs, are examining their neoplastic character and investigating their invasive potential [5,6,21,79,90]. They may be isolated, as fixed or fresh cells, and cultured to select for viable and proliferative

tumor cells [58]. Their targeted molecular characterization at the DNA, RNA and protein level in cancer patients is increasing insight into the biology of tumor cell dissemination and metastasis [2,5,7,9,21,72]. Harnessing detailed information on CTC biology will help to classify patients according to the invasive potential of their tumor cells, tailor drug selection for metastatic disease and monitor metastatic disease therapy. Clinical management of cancer patients should be improved by prognostic and predictive data derived from sequential measurements of therapy-induced changes in the pathology of CTCs/DTCs [2,5,7,9,20,36].

Genetic characterization of CTCs

Genetic abnormalities that have been detected in CTCs, including amplification and/or allelic loss of several oncogenes [91], dysfunctional telomerase activity [92] and aneuploid changes [93], are consistent with those in tumorigenic cells. Modern genetic techniques, such as gene expression profiling and whole-genome analysis, are being increasingly used to provide information on the molecular architecture of CTCs [5–7]. There is growing evidence that metastasis-capable malignancies have distinctive molecular footprints that can be detected in CTCs. The spectrum of early acquired genetic mutations and expression profiles may determine the metastatic ability of cancer cells [94], so informing the organ specific molecular identification of those CTCs having the highest metastatic potential. As dissemination progresses, only some of the heterogeneous and genomically unstable CTCs are thought to acquire genomic alterations representative of aggressive metastatic cells [2,20,57,95]. Since it is plausible that patients have CTCs with different metastatic potential (and sensitivity to treatment), their genetic and molecular characterization will help to assess the risk of tumor recurrence and define the therapy. Next-generation sequencing techniques provide an exciting platform for future detailed analysis of CTCs.

Defining tumor-specific DNA markers in CTCs of aggressive cancer phenotypes is one obvious goal of CTC research [36]. Owing to the substantial genetic heterogeneity of carcinomas, also within specific tumor types and individual tumor cells, multiple markers would need to be tested for sensitive DNA-based CTC screening protocols. Isolation and genetic characterization of single CTCs, as genomic irregularities in the primary tumor may not match those in

the disseminating cells [96], is feasible although technically challenging [5,7,8]. Using the ISET technique, Vona and colleagues presented the first convincing evidence that molecular characterization of CTCs could provide insight into the process of tumor invasion [37]. In their study of primary liver cancer, the rarity of β -catenin mutations in microdissected, single CTCs associated with disease progression, suggested that they had little involvement in initiation of tumor cell invasion. Cell-free DNA circulating in the blood has also been checked for tumor-specific anomalies and to signal the occurrence of DTCs in blood and BM in patients with prostate cancer [97,98].

Probes for gene targets of prostate and breast cancer therapies, for example *AR*, *EGFR*, *ERBB2* (*HER2*), *PTEN* and ETS-Related Gene (*ERG*), have been used in fluorescence *in situ* hybridization analysis of CTCs from advanced prostate cancer patients, and confirm that CTCs are malignant in origin [99,100]. Comparative genomic hybridization analysis arrays to identify copy number changes (i.e., gains and losses) in the genome of individual CTCs has been shown to enable prediction of whether breast cancer will remain localized or whether the patient will suffer metastases and disease relapse [58,95]. In metastatic prostate cancer, array comparative genomic hybridization profiles for CTCs were similar to paired solid tumor DNA [101].

qPCR expression markers

Gene expression profile analysis of CTCs by qPCR permits marker cut-off values for designating tumor cell-derived transcripts. Beyond their use in CTC detection techniques, these markers may help identify the invasive potential of CTCs and could even provide clues about the organ where the primary tumor mass is growing (in the absence of cancer diagnosis) [5,102].

Global gene-expression profile studies, such as for various clinical subtypes of breast cancers, have discovered new sensitive mRNA markers [103,104]. Metastatic gene expression signatures associated with DTCs in BM for genes involved in extracellular matrix remodeling, adhesion, cytoskeleton plasticity and signal transduction have been defined for breast cancer [41,94,102,105]. A list of cancer (CTC)-specific genes (such as *AGR2*, *S100A14*, *S100A16* and *FABP1*) was obtained from gene expression profiles of CTCs from metastatic CRC, prostate, and breast cancer patients, and their expression was used to differentiate between the cancers as well as normal

controls [106]. However, upregulation of common marker genes for CTCs often occurs in normal blood cells as a response to cytokine stimuli accompanying cancer progression and may occur in other conditions, such as chronic inflammatory disease, where CK20 mRNA levels are similar to patients with CRC cancer [107,108].

Various CTC detection methods using RT-PCR assays of targets, such as CK19, CK20, the EGF receptor (EGFR), CEA, human telomerase reverse transcriptase (hTERT), guanylyl cyclase C (GCC), and survivin have provided prognostic value in patients with breast, CRC, lung, melanoma, esophageal and head and neck cancers [74,109–116]. A recent RT-PCR based assay called 'AdnaTest' (AdnaGen AG, Germany) has been described for distinguishing CTCs expressing breast cancer gene transcripts following immunomagnetic separation of cells positive for MUC1, HER2 and EpCAM [96]. Besides the disadvantages of both EpCAM and RT-PCR-based protocols, the AdnaTest cannot enumerate CTCs and activated T lymphocytes also express MUC1 [117]. Altered expression of TWIST1, a transcription factor involved in EMT, was identified in DTCs from the BM of breast cancer patients following chemotherapy and was associated with early tumor relapse [118].

These, and other promising markers, need to be validated in large clinical studies, also including CK7 and MGB2 for breast cancer; squamous cell carcinoma (SCCA), EGFR and surfactant protein B (SFTPB) for lung cancer; TACS1 and SERPINB5 for colon cancer; transmembrane 4 super family 3 (TM4SF3) for gastrointestinal and prostate cancers; parathyroid hormone-related protein (PTHrP) and SCCA for head and neck cancer, and melanoma antigen gene protein A (MAGEA) and melanoma antigen recognized by T cells (MART)1 for melanoma [2,109,119]. The problems associated with the use of RT-PCR methods, discussed here, together with the heterogeneity of cancer subtypes and CTCs, indicate the requirement for multimarker mRNA panels and efforts to uncover more informative RNA markers continue [109,113]. In addition, circulating miRNAs represent a very promising new range of biomarkers to investigate [120].

Characterization of protein expression & signaling in CTCs

Expression changes in CTCs of assorted epithelial proteins (such as cytoskeleton-associated CKs, signaling kinases, growth factor receptors or surface adhesion molecules) may provide

organ-specific markers of increased metastatic potential [9,121–127]. For example, altered protein expression in CTCs of ERBB2, CK18, CK19, CK20, MUC1 and CEA may be used as specific biomarkers for breast cancer aggressiveness [23,128] (reviewed in [6]). A proliferative and survival advantage may be conferred on the subset of CTCs from breast cancers that express EGFR, HER2, PI3K, Akt, pFAK, hypoxia-inducible factor (HIF)1 α and VEGF phosphorylated receptors [129,130]. The EPISPOT method has indicated that CK19-releasing breast cancer cells possess high metastatic potential, where the detection by EPISPOT of full-length CK19 released by DTCs in BM correlated with the presence of overt metastasis and reduced survival [128]. This technique has also revealed that FGF2 (a stem cell growth factor) secreting CTCs represent prostate cancer cells with high metastatic properties [23].

Global proteomic analyses could be applied to CTCs as performed by 2D difference gel electrophoresis and mass spectrometry on DTC cell lines from the BM of breast cancer patients [131,132]. These cell lines express the EMT phenotype and protein profiles found in cancer stem cells, such as CD44⁺ and CD24⁻ [132]. Overall, investigations of CTC/DTC proteins show their expression to be pertinent for survival and maturation in distant organs. As noted previously, a number of the biological components of CTCs/DTCs are reminiscent of a cancer stem cell phenotype [42,46,133].

In summary, genetic and protein examinations of CTCs/DTCs have shed light on the mechanisms of cancer dormancy and metastasis, providing substantial evidence that they are active tumor cells with a definite heterogeneity in their metastatic potential [7]. Current focus is intensively directed at increasing this knowledge to locate biomarkers for biologically relevant therapeutic targets in metastatic progression, and to direct treatment choices at cancer diagnosis and during patient management.

Organ-specific therapeutic targets in CTC monitoring of minimal residual disease

Presently, the selection of patients for adjuvant therapies to prevent metastatic relapse depends on their estimated statistical risk of recurrent disease. This results in overtreatment with toxic agents in those patients where tumor cells have not disseminated from the primary tumor site. The emerging era of effective targeted cancer therapeutics of reduced toxicity encompasses a personalized approach based on the molecular

and biological properties of tumor subtypes [134]. As obtaining a tumor biopsy for biomarker assays of treatment prediction is often not possible or practical, CTCs have tremendous potential as an easily accessible ‘liquid biopsy’ of tumor-derived material. Furthermore, CTC profiling could actually more accurately reflect the patient’s current or metastatic disease than archived primary tumor tissue. Therefore, there is much current focus on how the early detection of CTCs, after primary tumor resection, together with a detailed understanding of the molecular and biological properties of these cells, will be integral in developing predictive biomarker assays to stratify patients for targeted systemic therapies (TABLE 1).

CTC biomarkers for monitoring adjuvant therapy

Currently, adjuvant therapy success can only be assessed retrospectively by the presence or absence of overt metastases during the postresection follow-up period. Biomarkers for instantaneous monitoring of adjuvant therapy efficacy are absolutely required as overt metastatic disease is presently incurable. Monitoring of CTCs during and after adjuvant therapy may aid clinical management of the individual cancer patient, permitting an early change in treatment before overt metastasis occurs. Critically, the molecular characterization of detected CTCs has the potential to offer personalized treatment selection in these most vulnerable patients (FIGURE 2).

Monitoring of CTCs in a Phase II trial (REMAJUS 02) of breast cancer, before and after neoadjuvant chemotherapy, established the presence of CTCs as an independent prognostic factor for decreased metastasis-free survival [85,135]. Efficacy of neoadjuvant chemotherapy (with or without trastuzumab, a monoclonal antibody to the ERBB2/neu receptor) is being addressed by the German Breast Group study GeparQuattro trial [133], and the large German SUCCESS trial of 1767 patients is focusing on adjuvant chemotherapy [136,137]. Ongoing, follow-up analyses of these trials will demonstrate any association between survival rates and decreases in CTC numbers. In approximately one fifth of patients, CTCs were detected before primary chemotherapy in the GeparQuattro trial and this rate halved following treatment ($p = 0.002$). Negating a straightforward gauge of treatment response, they did not observe an association between the response of the primary tumor to chemotherapy and CTC detection [133].

Table 1. Circulating tumor cell markers of prognosis, metastasis and therapy monitoring in the major carcinomas: circulating tumor cell counts as a prognostic tool for treatment response.

Study	Tumor type	Time of blood sampling/therapy test	Patients (n)	CTC detection/biomarker approach	Clinical value/main conclusion	Ref.
Rack <i>et al.</i> (2008) and (2010)	Primary breast cancer	Before and after chemotherapy	1500	CellSearch™	DFS, OS	[136,137]
Bidard <i>et al.</i> (2010) Pierga <i>et al.</i> (2008)	Locally advanced breast cancer	Before and after neoadjuvant chemotherapy (REMAGUS 02 trial)	115	CellSearch	PFS, OS (before chemotherapy), relapse	[84,135]
Cristofanilli <i>et al.</i> (2004) Hayes <i>et al.</i> (2006)	Metastatic breast cancer	Before and after chemotherapy	177	CellSearch	PFS, OS	[47,49]
Bidard <i>et al.</i> (2010)	Metastatic breast cancer	After chemotherapy + bevacizumab (French substudy of MO19391 trial)	67	CellSearch	TtP; treatment course may modify the predictive value of CTCs	[84,85]
Tol <i>et al.</i> (2010)	Advanced colorectal cancer	Before and after chemotherapy plus targeted agents (CAIRO2 trial)	467	CellSearch	PFS, OS	[172]
Cohen <i>et al.</i> (2008)	Metastatic colorectal cancer	Before and after chemotherapy	430	CellSearch	PFS, OS	[81]
de Bono <i>et al.</i> (2008)	Castration-resistant prostate cancer	Before and after chemotherapy	231	CellSearch	OS	[80]
Hofman <i>et al.</i> (2010)	Non-small-cell lung cancer	Before and after surgery	208	ISET™	OS, DFS	[86]

Examples of some important trials of CTC counts as a prognostic tool for treatment response.

CTC: Circulating tumor cell; DFS: Disease-free survival; OS: Overall survival; PFS: Progression-free survival; TtP: Time to progression.

CTC biomarkers relevant to metastatic disease & targeted anticancer therapies

As discussed previously, the biological properties of the primary tumor and its disseminating cell offspring can diverge. This reinforces the constraint in examining only primary tumor cells and the importance of characterizing the metastatic cells. Experiments demonstrate that most DTCs and CTCs from patients with breast, colon and lung cancer are proliferation antigen Ki-67 negative, and appear to be non- or slow-proliferating cells, indicating chemotherapy resistance [20,42,56]. However, use of the CTC-chip in metastatic prostate cancer indicates that, depending on disease evolution during therapy, the proliferative abilities of CTCs vary widely (from 1 to 81% using a Ki-76 score) [27]. Developing targeted therapies in concert with established chemotherapy and radiotherapy regimens to eradicate dormant or noncycling CTCs is a major challenge and goal for oncologists.

Coupling CTC detection to organ-specific marker characterization in monitoring of targeted therapies promises exciting clinical

advancements in cancer treatment. Provisional data from clinical trials of several chemotherapeutic agents and novel targeted therapies strongly indicate that CTC analyses offer vital prognostic information [7,112,133,137,138]. Further investigations and extended follow-up times will demonstrate to what extent changes in CTC numbers and biology can comprehensively predict response to particular therapies.

Thereafter, most of the key developments in the use of CTCs for cancer markers of metastatic disease have been performed in breast cancer (reviewed in [6,64]), and many examples of this work have been discussed in this article. The following section describes some examples of the most currently promising cancer specific CTC biomarkers, with a particular focus on ERBB2 in breast cancer, to illustrate this potential (TABLE 2).

Breast cancer

One of the most exciting developments in CTC research has been in the use of the receptor tyrosine kinase *ERBB2* (*HER2*) proto-oncogene as a therapeutic target in the monitoring of breast

cancer metastasis. Overexpression of the protein product owing to *ERBB2* gene amplification is found in approximately one quarter of breast cancers and is associated with aggressive invasive features and impaired prognosis [46,139]. Clinical trials of systemic therapy in ERBB2-positive breast tumors using a monoclonal antibody (trastuzumab) against ERBB2 demonstrate significantly improved disease-free survival and OS [140–142].

Although some studies have reported that ERBB2 status is equivalent between DTCs/CTCs and the majority of corresponding primary tumors [36,143], several investigators have detected DTCs and CTCs expressing ERBB2 in some patients with ERBB2-negative primary tumors [36,133,139,144–148]. These differences may be explained by failure of the fluorescence *in situ* hybridization analysis to signal the existence of a small subclone of *ERBB2*-amplified cells in the primary tumor with a potential to disseminate and/or development of *ERBB2* gene amplification in DTCs/CTCs [146]. Several other factors could account for these differences, including the applicability of the methods used to capture the relevant CTCs, that ERBB2 status, determined by immunofluorescence, is not a standardized assay, and the numbers of CTCs needed to adequately assess ERBB2 status in a heterogeneous sample [36]. Nevertheless, as ERBB2 definition on the primary tumor is problematic and is usually a once-only assay, the detection of ERBB2-positive CTCs would provide a real-time evaluation of the ERBB2 status during the clinical disease case [36,133]. In a further study of 431 patients with primary breast cancer typed by RT-PCR, CTCs were mainly found to be triple-negative, regardless of the ERBB2, estrogen receptor (ER) and progesterone receptor (PR) status of the primary tumor [96]. This difference in the expression profile between CTCs and the primary tumor has implications for the selection of adjuvant therapy and the biology of the primary tumor appears to influence the distribution of CTCs. Indeed, a more general phenomenon of disparity in the genome and protein properties of some CTCs from the primary tumor may well impact on how tumor cells respond to therapy, possibly explaining why trials such as REMAGUS 02 and GeparQuattro did not observe a significant association between CTC number and therapeutic response of the primary breast tumor [36,85,133,135]. Intriguingly, in the study by Meng and colleagues, Herceptin® (Genentech Inc., CA, USA; trastuzumab) therapy was beneficial in three out of four patients with ERBB2-positive CTCs, whose

primary tumors were ERBB2-negative [146]. The resounding message from these efforts is that more patients may benefit from ERBB2-directed therapies [148].

An important question to be answered by current clinical studies is whether the ERBB2 status of CTCs or DTCs can predict efficacy of ERBB2-directed therapies [133,148]. Whether anti-ERBB2 therapy can be enhanced by new regimens (lapatinib and/or trastuzumab) with subsidiary analysis of CTCs, is being addressed by the Adjuvant Lapatinib And/Or Trastuzumab Treatment Optimisation (ALTTO) clinical study [149]. Furthermore, upregulation of the chemokine receptor CXCR4 is critical for ERBB2-mediated metastasis [150]. As CXCR4 facilitates cancer cell motility, primarily to BM, the link between ERBB2 and CXCR4 signaling may explain the increased detection rate of ERBB2-positive DTCs in BM and peripheral blood [139,144]. The expression of CXCR4 receptors and improved angiogenic capacity of these cells are additional features of high metastatic capability [40,151]. Thus, CXCR4 represents a promising marker for breast cancers of high metastatic likelihood, and antibodies directed against CXCR4 are in preclinical development.

The same RT-PCR markers frequently used in CTC detection protocols also demonstrate promise as indicators of MRD. For example, in early breast cancer, the presence of CK19 mRNA-positive CTCs, detected by RT-PCR,

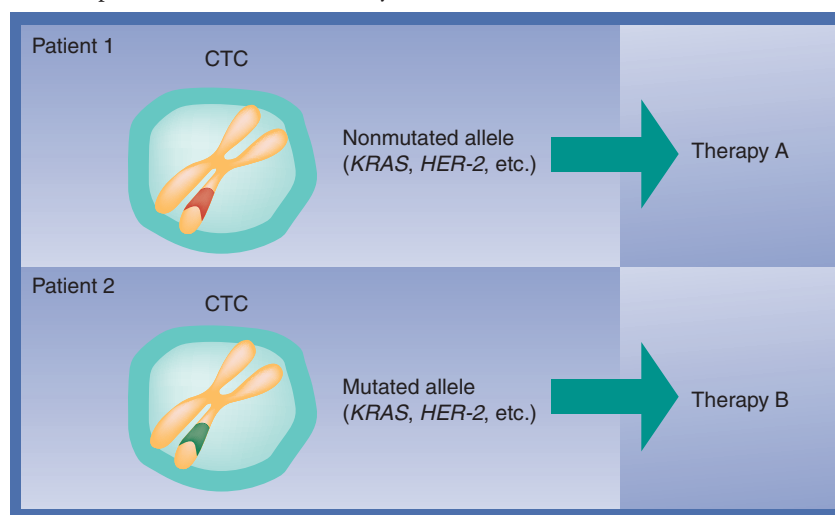


Figure 2. How circulating tumor cell biomarkers may be used to direct cancer therapies to the individual cancer patient. In this schematic, therapy is chosen based on the genetic status of *KRAS* or *HER-2* (*ERBB2*) in CTCs. This may provide added clinical information regarding disease aggressiveness and/or indicate patient suitability for specific targeted therapies. CTC: Circulating tumor cell. Reproduced with permission from [203].

Table 2. Circulating tumor cell markers of prognosis, metastasis and therapy monitoring in the major carcinomas: circulating tumor cell molecular markers.

Study	Tumor type	Time of blood sampling/therapy test	Patients (n)	CTC detection/biomarker approach	Clinical value/main conclusion	Ref.
Breast cancer						
Quintela-Fandino et al. (2006)	Advanced breast cancer	After surgery and adjuvant chemotherapy before high-dose chemotherapy	84	CK19, ERBB2, PTB, PS2, EGP2 multimer RT-PCR	DFS, OS	[45]
Reithdorf et al. (2010)	Primary breast cancer	Before and after neoadjuvant chemotherapy (and ± trastuzumab) in GeparQuattro trial	213	CellSearch™/ERBB2 expression immunoscore	Stratification and monitoring of ERBB2-directed therapies	[133]
Meng et al. (2004)	Primary breast cancer	Postadjuvant therapy	31	Immunicon™/ERBB2 FISH	Trastuzumab therapy beneficial in ERBB2 + CTC/primary tumor ERBB2	[146]
Fehm et al. (2009)	Primary breast cancer	Before adjuvant therapy	431	AdnaTest BreastCancer™(EpCAM, MUC1 and ERBB2 transcripts)/RT-PCR (ER, PR expression)	Selection of adjuvant treatment	[96]
Wulfig et al. (2006)	Primary breast cancer	Before surgery	42	Immunomagnetic separation/immunocytochemistry ERBB2 status	DFS, OS	[139]
Ignatiadis et al. (2007)	Primary breast cancer	Before chemotherapy	167, 444	CK19 RT-PCR	DFS, OS	[152]
Xenidis et al. (2009)	Primary breast cancer	Postadjuvant chemotherapy	437	CK19 RT-PCR	DFS, OS	[138]
Xenidis et al. (2007)	Primary breast cancer	Adjuvant tamoxifen	119	CK19 RT-PCR	DFS, OS	[112]
Fehm et al. (2010)	Metastatic breast cancer	After metastatic disease	254	CellSearch/AdnaTest BreastCancer	CTC ERBB2 status impacts on selection of adjuvant treatment	[148]
Rack et al. (2010)	Primary breast cancer	Before and after adjuvant chemo in SUCCESS trial	1767	CellSearch/anti-CK8,18,19 and anti-CD45 antibodies	LN metastases (before chemotherapy); DFS, OS (before chemotherapy); DFS (after chemotherapy)	[137]
Kallergi et al. (2009)	Metastatic breast cancer	After metastatic disease	34	Confocal laser scanning microscopy/immunomagnetic-separation/CK19, VEGF, VEGF2, HIF1α, pFAK expression	Metastatic therapeutic targeting	[129]
Yie et al. (2006)	Primary breast cancer	Before adjuvant chemotherapy	67	Survivin RT-PCR ELISA	Relapse	[156]
Shen et al. (2009)	Primary breast cancer	Before surgery and therapy	94	Survivin, hTERT, hMAM multimer RT-PCR	Disease progression/metastatic potential	[173]
Promising organ-specific metastatic potential and treatment response CTC biomarkers: AR: Androgenic receptor; CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; CTC: Circulating tumor cell; DFS: Disease-free survival; EGFR: EGF receptor; ER: Estrogen receptor; FISH: Fluorescence in situ hybridization; HIF: Hypoxia-inducible factor; hMAM: Human mammaglobin; hTERT: Human telomerase reverse transcriptase; IHC: Immunohistochemistry; LN: Lymph node; MUC: Mucin; OS: Overall survival; PFS: Progression-free survival; PR: Progesterone receptor; PSA: Prostate-specific antigen; PT-PCR: Reverse transcription PCR; Tsp: Time to progression						

Table 2. Circulating tumor cell markers of prognosis, metastasis and therapy monitoring in the major carcinomas: circulating tumor cell molecular markers.

Study	Tumor type	Time of blood sampling/therapy test	Patients (n)	CTC detection/biomarker approach	Clinical value/main conclusion	Ref.
Gastrointestinal cancer						
Wong et al. (2009)	CRC	Before and after surgery	132	Immunomagnetic enrichment/CK20 immunocytochemistry	Relapse, OS	[160]
Wang et al. (2006)	CRC	Before surgery, after surgery	72, 157	hTERT, CK19, CK20, CEA multimer RT-PCR	Relapse, survival	[110,113,116]
Wang et al. (2007)	CRC	Before surgery, after surgery	72, 157	hTERT, CK19, CK20, CEA multimer RT-PCR	Relapse, survival	
Gervasoni et al. (2008)	CRC	Before surgery	34	CK20 IHC/c-MET, CK20, MAGE-A3, GaINAc-T multimer RT-PCR	DFS	[174]
Uen et al. (2008)	CRC	Before and after surgery	438	Membrane array/hTERT, CK19, CK20 and CEA multimer RT-PCR	Relapse	[175]
Yen et al. (2009)	Metastatic CRC	During chemotherapy plus cetuximab	76	KRAS membrane array	Predicts response to cetuximab, PFS, OS	[161]
Yie et al. (2008)	Colorectal and gastric cancer	After surgery, before adjuvant therapy	86 (CRC) 55 (gastric)	Survivin RT-PCR ELISA	Relapse	[157]
Cao et al. (2009)	Esophageal squamous cell carcinoma	Before surgery, before chemotherapy	108	Survivin RT-PCR ELISA	Relapse, survival	[158]
Wu et al. (2006)	Gastric cancer	During resection or palliative surgery	64	Colorimetric membrane-array/CK19, hTERT, CEA, MUC1 multimer RT-PCR	Relapse, survival	[159]
Prostate cancer						
Alix-Panabieres et al. (2007)	Localized prostate cancer	After cancer diagnosis	19	EPISPOT/FGF2, CK19, MUC1 secretion	Metastatic potential	[23]
de Bono et al. (2007)	Metastatic prostate cancer	Before and after chemotherapy or chemotherapy + anti-IGF-IR antibody	80	CellTracks (Immunicon)/IGF-IR immunofluorescence	Monitoring of anti-IGF-IR therapy in hormone-refractory prostate cancer	[155]
Shaffer et al. (2007)	Metastatic prostate cancer	After metastatic disease	63	CellSearch/EGFR expression, chromosome ploidy, AR gene amplification	EGFR and AR CTC profiling may have a role in clinical management	[99]
Stott et al. (2010)	Localized and metastatic prostate cancer	Before and after resection/chemotherapy	19 (local) 36 (metastatic)	CTC chip/PSA staining, Ki67 staining, TMPRSS2-ERG RNA fusion	Platform to test clinical studies of CTCs in invasive localized disease and targeted therapies for metastatic disease	[27]
Reid et al. (2010)	Castration-resistant prostate cancer	After chemotherapy and before and after abiraterone acetate treatment (inhibits CYP17)	34	CellSearch/ERG rearrangements, PSA	Decline in CTCs is evidence of antitumor effects of abiraterone acetate	[153]

Promising organ-specific metastatic potential and treatment response CTC biomarkers.
 AR: Androgenic receptor; CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; CTC: Circulating tumor cell; DFS: Disease-free survival; EGFR: EGF receptor; ER: Estrogen receptor; FISH: Fluorescence in situ hybridization; HIF: Hypoxia-inducible factor; hMAM: Human mammary factor; hTERT: Human telomerase reverse transcriptase; IHC: Immunohistochemistry; LN: Lymph node; MUC: Mucin; OS: Overall survival; PFS: Progression-free survival; PR: Progesterone receptor; PSA: Prostate-specific antigen; RT-PCR: Reverse transcription PCR; Ttp: Time to progression.

Table 2. Circulating tumor cell markers of prognosis, metastasis and therapy monitoring in the major carcinomas: circulating tumor cell molecular markers.

Study	Tumor type	Time of blood sampling/therapy test	Patients (n)	CTC detection/biomarker approach	Clinical value/main conclusion	Ref.
Lung cancer						
Maheswaran et al. (2008)	Metastatic non-small-cell lung cancer	After diagnosis and during therapy regimens	27	CTC chip/EGFR DNA mutations	Therapy response, tumor progression	[26]
Chen et al. (2007)	Advanced non-small-cell lung cancer	Before and after chemoradiation	67	CK19 RT-PCR	PFS, OS	[111]
Yie et al. (2009)	Non-small-cell lung cancer	Before surgery, before chemotherapy	143	Survivin RT-PCR ELISA	Relapse, survival	[163]
Promising organ-specific metastatic potential and treatment response CTC biomarkers. AR: Adrenergic receptor; CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; CTC: Circulating tumor cell; DFS: Disease-free survival; EGFR: EGF receptor; ER: Estrogen receptor; FISH: Fluorescence in situ hybridization; HIF: Hypoxia-inducible factor; hMAM: Human mammaglobin; hTERT: Human telomerase reverse transcriptase; IHC: Immunohistochemistry; LN: Lymph node; OS: Overall survival; PFS: Progression-free survival; PR: Progesterone receptor; PSA: Prostate-specific antigen; RT-PCR: Reverse transcription PCR; Ttp: Time to progression.						

predicted poorer outcome in ER-negative, triple-negative and ERBB2-positive subgroups, prior to adjuvant chemotherapy [152]. In the same patients, CK-19 mRNA-positive CTCs, postadjuvant chemotherapy, indicated the presence of resistant residual disease as these patients had significantly reduced OS and disease-free survival [138].

Finally, antiangiogenic drugs, such as the monoclonal antibody bevacizumab (Avastin™ [Genentech Inc., CA, USA]), targeted against VEGF, may help maintain the nonproliferative state of CTCs and DTCs [72]. As VEGF and VEGF receptor 2 were expressed on approximately 70% of all the detected CTCs in a recent study of metastatic breast cancer, these proteins represent potential therapeutic targets [129].

Prostate cancer

The tumor suppressor activity of abiraterone acetate, which inhibits *CYP17* (central to androgen metabolism in the prostate tissue, testes, and adrenal glands), was appraised in a Phase II trial of docetaxel-treated patients with castration-resistant prostate cancer. A decrease in CTC numbers following treatment in the majority of patients suggests abiraterone acetate has considerable antitumor effects [153,154]. Clinically useful information was provided by the CTC counts in addition to blood serum levels of PSA, the major biomarker for prostate cancer [154]. There was also a significant correlation observed between PSA and CTC count declines in patients with *ERG* gene rearrangements in their tumors [153]. Immunofluorescent detection of CTCs expressing IGF-IR has shown promise in a preliminary clinical study as a biomarker for advanced disease in prostate cancer patients undergoing anti-IGF-IR antibody therapy (targeted to a different IGF-IR epitope) [155]. Expression of *EGFR* and adrenergic receptor (*AR*) gene amplification profiling of CTCs is currently being assessed for the clinical management of patients with castration-resistant prostate cancer. In the feasibility study, molecular profiling of the CTCs appeared to provide better insight into the biological heterogeneity of the disease than tumor site biopsies [99]. The prostate cancer-specific *TMPRSS2-ERG* fusion can be detected in RNA from CTCs and was used in a pilot study (along with PSA) as a marker associated with metastatic prostate cancers [27]. This platform demonstrates promise to facilitate targeted therapies monitored by the *TMPRSS2-ERG* and PSA biomarkers.

Gastrointestinal cancers

The relationship of RT-PCR assays of hTERT, CK19, CK20 and CEA to postoperative metastatic relapse were examined in CTCs from CRC patients and healthy controls. Only CEA was estimated to be an independent significant predictor of postoperative metastasis and is currently being further evaluated for its use in early detection of micrometastatic CTCs in CRC patients [110]. The detection of CTCs expressing survivin (an apoptosis inhibitor) by an RT-PCR enzyme-linked immunosorbent assay, developed by Yei and colleagues, provided important predictive information for breast cancer metastasis and recurrence [156]. The same team then used this technique to demonstrate that survivin expression in CTCs from patients with gastric cancer, CRC and esophageal squamous cell carcinoma was associated with disease stage, decreased survival and a higher relapse risk [157,158].

Oligonucleotide probes and alkaline phosphatase detection of CTC target genes have been used in colorimetric membrane-array techniques avoiding cell enrichment and qPCR. These assays involve amplification of total peripheral blood RNA, cDNA synthesis and hybridization to membrane arrays to enable quantification of binding intensities. Therefore, as for all exclusively mRNA-based detection methods, it cannot be certain to what degree CTCs are the source of the genetic variation. A preliminary study on gastric cancer patients and healthy controls used this approach to simultaneously detect hTERT, CK19, CEA and MUC1 [159]. The four marker combination had a diagnostic accuracy of approximately 90% and was a significant independent predictor for disease recurrence and metastasis. Wong and coworkers developed and tested a gastrointestinal-specific anti-CK20 antibody to detect CTCs in CRC patients [160]. A comparison of chromosome 17 aneusomy between the primary tumors and CK20⁺ CTCs with 90% concordance confirmed the malignant nature of these cells. Furthermore, CTC counts of CK20⁺ cells were associated with survival, disease recurrence and metastasis.

A high-throughput method for detection of *KRAS* mutations by a membrane array was used to demonstrate the clinical application of *KRAS* oncogene characterization in CTCs for predicting cetuximab response in patients with metastatic colorectal cancer [161]. There was a high concordance observed between *KRAS* status of the tumors and CTCs, and mutations in both were strongly associated with response to cetuximab,

progression-free survival and OS ($n = 86$; $p < 0.0001$). An enhancement of the sensitivity of this technique, using chemiluminescence, has recently been reported [162].

Lung cancer

Drug-resistant variants of the *EGFR* gene limit the use of tyrosine kinase inhibitors of EGFR in cancer therapy. Genetic analysis of *EGFR* mutations in DNA isolated from CTCs (by the CellPoint [CO, USA] CTC Chip platform™ [On-Q-ity, MA, USA]) of metastatic lung cancer patients were used to monitor treatment response to gefitinib (Iressa), an EGFR inhibitor [26]. Longitudinal analysis of CTC-derived genotypes indicated the molecular evolution of the tumor during therapy. Importantly, this study demonstrated the feasibility of using blood samples rather than tumor biopsies for monitoring tumor cell genotypes in response to treatment. The detection of survivin-expressing CTCs by a RT-PCR ELISA assay in NSCLC patients correlated with disease stage and was an independent predictor for cancer recurrence and survival [163].

Non-epithelial cancer

Techniques to isolate and characterize CTCs have primarily focused on their occurrence in epithelial cancer patients. Interestingly, CTCs were recently successfully isolated by ISET in a study of 87 patients with either primary cutaneous invasive melanoma or metastatic melanoma [164]. To add additional power to morphologically distinguishing large monocytes from smaller melanoma cells, the CTCs isolated on the ISET filters were able to be characterized by immunohistochemical markers (S100 protein, human melanoma black [HMB]-45, MART-1 or CD45) and by RT-PCR of tyrosinase mRNA. As CTC detection was markedly associated ($p = 0.001$) with the presence of tyrosinase mRNA in blood samples, RT-PCR assays of tyrosinase on single isolated CTCs could aid monitoring of therapies in melanoma patients.

In summary, personalized therapy against MRD may be improved by defining the selection of patients for specific directed treatments based on the molecular status of CTCs. Moreover, additional real-time evaluation of CTCs could monitor treatment induced tumor cell alterations and provide a novel method to gauge further targeted therapies [2,7,36]. Currently, several new markers detected in CTCs are being tested in pharmacodynamic studies and show promise as indicators of metastasis capable malignancies.

Conclusion

There is now sufficient evidence to conclude that CTCs in the blood play an important role in the metastatic process and can be detected years before the occurrence of distant overt metastases. They provide a means to investigate the biological features of micrometastatic cells in patients with early-stage cancer and a diagnostic and prognostic source in metastatic cancer patients.

Particularly in breast cancer, the demonstrated clinical relevance of CTC detection in early-stage disease could be used for individualized risk assessments superior to tumor, node, metastases (TNM) staging. However, owing to the discordant results with different CTC detection modalities, there is a requirement for well-standardized methods across different laboratories and improved identification of CTCs with an EMT phenotype to enhance the clinical benefit in early-stage cancer patients. Thus, the prognostic relevance of CTCs in early-stage cancer patients requires further clarification in prospective multicenter trials.

Nevertheless, the sensitive and specific detection of relevant CTCs has huge potential to direct early treatment strategies to avoid recurrence and metastases, and their isolation permits monitoring of systemic tumor cell dissemination in the blood. Detailed screening and molecular characterization of these cells should increase insight into the mechanisms of metastatic dissemination and improve prediction and monitoring of treatment efficacy. Although most of the existing evidence highlights the role of CTCs in metastatic cancer patients, CTC profiling appears also to be relevant for clinical management in (neo) adjuvant therapy. A prominent clinical application of CTCs is to monitor minimal residual disease in patients with no signs of overt metastasis and identify those individuals at increased risk for recurrence as additional treatments may aid these patients. Ongoing prospective clinical studies are assessing whether the elimination of CTCs in the blood with (neo)adjuvant treatments and therapy administered, based on CTC biology for metastatic cancer patients, associates with improved outcomes.

Circulating tumor cells comprise a heterogeneous group with biological properties often distinct from their primary tumor cell counterparts. Knowledge of the molecular architecture of CTCs is accessing novel organ specific markers that improve information regarding tumor stage, metastatic capability, progression

and response to therapy. These data may enable, in individual cancer cases, a tailored choice of therapeutic strategies to prevent recurrence and metastatic relapse. For example, accumulating evidence illustrates how ERBB2 status can alter during breast cancer progression or recurrence and the reassessment of ERBB2 status in CTCs offer promise of a breast cancer specific marker for improved management of metastatic disease [148]. The ability of CTCs to survive current chemotherapy may provide an early indicator of treatment inadequacy, allowing modification of curative strategies. Specific CTC biomarkers may further help calibrate the effectiveness of therapy and indicate when and what alternative therapies should be considered, particularly where the clinical and radiographic information is ambiguous. Together, this will provide a powerful approach to better prevent cancer invasion. Overall, CTC molecular biomarkers are generally not yet ready for routine use and need to be rigorously clinically validated. However, currently, sequential monitoring of CTCs in blood samples would provide time-point estimates of therapeutic efficacy and clearance of CTCs could function as an intermediate end point in clinical trials of anticancer agents.

In summary, the detection and molecular characterization of CTCs provides important prognostic indicators and a means for individualized prediction and real-time monitoring of the efficacy of systemic therapy. Detailed molecular investigations of CTCs will increasingly help define organ-specific biomarkers as early predictors of metastasis-capable malignancies and the development of new targeted biological therapies.

Future perspective

The isolation and molecular characterization of CTCs has provided new insights into the intricacies of the metastatic process with the promise of improved therapeutic approaches. However, to translate these findings into routine clinical practice, major challenges need to be addressed. Particularly in early-stage patients, the few tumor cells detected by existing methods limits assay robustness, compounded with the lack of adequate standardization and leukocyte contamination problems for both detection and the downstream tumor cell molecular profiling techniques.

Current investigations are trying to decipher whether CTCs from various epithelial tumors can use the common homing organ of the BM to survive chemotherapy and persist for

years in a quiescent state. Ongoing clinical trials are examining the clinical utility of CTCs for disease monitoring after tumor resection. Low counts hinder the value of CTC analysis for early-stage cancer patients and perhaps a substantial number of these cells undergoing EMT are overlooked by current commonly used EpCAM-based CTC detection methods. Indeed, there is a need for greater focus on identifying markers to isolate and characterize this subpopulation of CTCs with EMT and tumor stem cell characteristics proposed to be the founder cells of overt metastases. Key challenging research avenues are to decipher the molecular mechanisms behind this quiescent state, what activates proliferation of these tumor cells and to identify the initiator cells (metastatic stem cells) of overt metastases [2,142,165]. An increased understanding of how these cells interact with other molecular and cellular process (such as genes and miRNAs involved in metastasis promotion and inhibition [142,166,167]) added to the contribution of host genetic background [168] in forming micrometastases is a formidable challenge. This will necessitate functional *in vitro* and *in vivo* studies (including xenotransplanted mouse models that mimic MRD) to measure the biological properties of CTCs from findings in cancer patients and to identify metastatic competent CTCs. To investigate the role of circulating cancer stem cells in metastasis, further experiments should examine the correspondence of markers of cancer stem cells and their levels

in CTCs. For example, recent evidence suggests that a CD26⁺ subpopulation of CD133⁺ cancer stem cells, isolated from primary CRCs and liver metastatic tumors, may serve as a strong predictive marker for metastasis [169]. Furthermore, these cells (but not CD26⁻ cells) formed liver metastasis when injected into the cecal wall of mice. Studies on the consequences of resection technique for tumor cell dissemination are also required, as addressed by an ongoing prospective randomized multicenter trial using RT-PCR for CK20⁺ CTCs to examine conventional versus anterior hepatic resection in metastatic CRC patients [170].

Enumeration and molecular characterization of CTCs has increasing merit for tailored selection of patients to systemic therapies (such as ERBB2 or EGFR antagonists) and biomarkers of response during treatment monitoring. There is now the exciting possibility that this knowledge of CTC biology will identify metastatic tumor-specific targets to enhance therapy regimens. Soon, CTC detection and characterization may serve as a real-time 'liquid biopsy' to continually refine prognosis and tailor anticancer therapy to the individual patient. Prospective randomized studies are currently evaluating whether therapy steered by CTC testing can improve the outcome of metastatic patients and whether the eradication of CTCs in the blood is associated with a longer disease-free stage and OS. Biomarkers for the circulating tumor stem cells among the CTCs are needed

Executive summary

Circulating tumor cell clinical relevance

- There is much current effort to examine the clinical relevance of circulating tumor cell (CTCs) for prognosis and monitoring individual patient response to systemic therapies.
- In metastatic cancer CTCs are clearly a strong prognostic indicator.
- Examining CTCs in the peripheral blood of cancer patients could render the invasive examination of repeated bone marrow aspirations for disseminated tumor cells (DTCs) unnecessary.
- The use of CTC assays has been particularly focused on the metastatic setting, although there is additional relevance for use in administering adjuvant systemic therapy.

Metastatic mechanisms

- The molecular characterization of CTCs has greatly improved knowledge of the metastatic process.
- Biomarkers and therapies need to target the cancer stem cells among the CTCs and research to identify circulating tumor stem cells is ongoing.

Targeted therapy & molecular biomarkers of metastatic potential

- Molecular characterization of CTCs offers new approaches for individualized therapeutic targeting for cancer patients to supplement chemotherapy and radiotherapy.
- Optimal antimetastatic therapy in cancer patients may derive from assessing organ specific therapeutic targets on CTCs.
- ERBB2 is a promising marker to direct additional systemic therapy for breast cancer.
- CTC biomarkers can be used for real-time monitoring in individual cancer patients of systemic therapy efficacy.
- CTC biomarkers can serve as early predictors of metastasis-capable malignancies and aid development of new targeted therapies.
- To improve targeting of therapies to tumors that express the target will be greatly facilitated by CTC-based biomarker assays that provide reliable, real-time expression information.

to direct therapeutic targeting and for important prognostic indicators of tumor cells, resistant to therapy [171]. Current promising markers now require thorough validation in large, clinical studies, for example, to estimate the association of ERBB2-positive CTCs in metastatic cancer with response to ERBB2-targeted therapy. Future trials of targeted therapies to metastatic cancer cells should also consider their affect on CTCs, to better select patients for a particular regimen and to examine properties of cells that can survive therapy. For instance, the therapy may select tumor cells not expressing the target and/or those that do express the target but have evolved drug resistance mechanisms, such as resistance to trastuzumab resulting from downstream signaling pathway mutations [7,133].

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