Review Article

CTC enumeration and characterization: moving toward personalized medicine

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Abstract: The primary cause of tumor-related death in breast cancer (BC) is still represented by distant metastasization. The dissemination of tumor cells from the primary tumor to distant sites through bloodstream cannot be early detected by standard imaging methods. The enumeration of circulating tumor cells (CTCs) represents an effective prognostic and predictive biomarker, which is able to monitor efficacy of adjuvant therapies, detect early development of (micro)metastases and at last, assess therapeutic responses of advanced disease earlier than traditional imaging methods. Moreover, since repeated tissue biopsies are invasive, costly and not always feasible, the assessment of tumor characteristics on CTCs, by a peripheral blood sample as a 'liquid biopsy', represents an attractive opportunity. The implementation of molecular and genomic characterization of CTCs could contribute to improve the treatment selection and thus, to move toward more personalized treatments. This review describes the current state of the art on CTC detection strategies, the evidence to demonstrate their clinical validity, and their potential impact for both future clinical trial design and, decision-making process in our daily practice.

Keywords: Breast cancer (BC); circulating tumor cells (CTCs); liquid biopsy; precision medicine

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Introduction

Breast cancer (BC) remains the most common type of cancer diagnosed among women and is responsible for 15% of all cancer-related deaths with 40,000 estimated deaths in 2014 (1). Although the improvements in BC detection and adjuvant treatments led to a significant decrease in BC-related deaths in the last two decades, about 30% of women initially diagnosed with early-stage cancer eventually develop metastatic disease. Moreover, despite advances in the treatment of metastatic BC, at this point it remains practically incurable, and the aims of therapy are the prolongation of overall survival (OS) time and the improvement of quality of life (2).

The leading causes of tumor-related death in BC remain the complications from distant metastasization. Unfortunately, the spread of tumor cells through haematogenous dissemination from the primary tumor to distant sites cannot be detected by standard imaging methods. Therefore, there is an urgent need to find novel biomarkers, which could monitor efficacy of adjuvant therapies, detect early development of (micro)metastases and at last, assess therapeutic responses of advanced disease. In the last decade, the detection of disseminated tumor cells (DTCs) in the bone marrow and circulating tumor cells (CTCs) in the blood have demonstrated to provide useful information for the clinical management of BC by predicting treatment benefit earlier than traditional imaging methods.

More recently, other potential blood-based markers are emerging as independent parameters for prediction development and outcome in metastatic disease, including circulating tumor microemboli and circulating tumor materials (CTMat). The apoptosis and necrosis processes of CTCs cause the leakage of intracellular components in the bloodstream, such as electrolytes, cellular debris, DNA, and chromatin. Since CTCs are continuously released and destroyed, such CTMat accumulate and could represent an independent biomarker for the prognostication and

Table 1 Advantages and disadvantages of the main enrichment and detection techniques

Technique	Advantages	Disadvantages	References
CTC-filtering devices by size (ISET)	Capture and analysis platform; multiplexed imaging and genetic analysis; easy and rapid; feasible for EpCAM-negative CTCs	Low specificity (lose smaller CTCs and retain larger leukocytes)	(3-5)
Density gradient centrifugation (Ficoll-hypaque or OncoQuick)	Easy and inexpensive; feasible for EpCAM-negative CTCs	Low specificity; cross-contamination of different layers (OncoQuick can resolve this issue)	(6,7)
CellSearch® system	FDA cleared; visual confirmation of CTCs; clinical relevance; automated, quantitative; highly reproducible	EpCAM-positivity dependent; no additional gene expression tests could be added for analysis of CTCs; subjective picture evaluation; costly instrumentation	(8,9)
CTC-chip	High detection rate; visual confirmation of CTCs; potential to harvest CTCs for further molecular and genetic analyses	EpCAM-positivity dependent; subjective CTC analysis; further investigation on assay specificity	(10,11)
Immunocytochemistry (ICC)	Quantification and morphological analysis of CTCs; facilitate classical cytopathological review	Time-consuming; subjective evaluation	(12)
Protein assays (EPISPOT)	Detects only viable cells; limited number of markers	Clinical relevance not demonstrated; proteins must be actively secreted; no further identification and isolation of CTCs	(13,14)
Immunofluorescence-based technologies (DyLight)	Multimarker image analysis	Application in cell lines	(15)
RT-PCR (CTCscope)	High sensitivity; detects only viable cells	No morphological analysis; visualization and enumeration of CTCs is not possible	(16)
Multiplex RT-PCR (AdnaTest)	High sensitivity; detects only viable cells; saves sample and time, reduces cost; isolation and detection of stem cell and EMT markers	No morphological analysis; EpCAM and MUC1 positivity dependent assay; no quantification	(17,18)

ISET, isolation by size of epithelial tumor cells; CTCs, circulating tumor cells; EPISPOT, EPithelial ImmunoSPOT; EMT, epithelial mesenchymal transition; EpCAM, epithelial cell adhesion molecule.

monitoring of metastatic disease.

The aim of this review is to describe the current state of the art on CTCs detection and clinical use, the evidence to demonstrate their clinical validity, and their potential impact for both future clinical trial design and, decision-making process in our daily practice.

Strategies for CTC analysis

CTCs are present in the bloodstream at a very low concentration, thus their detection and characterization require highly sensitive and specific methods, which consist of a combination of enrichment (isolation) and detection (identification) strategies (*Table 1*) and both steps are essential components of the identification process (19).

CTC enrichment

CTC enrichment strategies are based on technologies that can distinguish CTCs among the surrounding hematopoietic cells, according to their physical (size, density, electric charges, deformability) and biological (cell surface protein expression, viability) characteristics. Therefore, enrichment techniques are based on two different strategies:

the selection according to morphological features or according to immunologic profile (12).

Several membrane filter devices are available for CTC enrichment based on the differential cellular size, including isolation by size of epithelial tumor cells (ISET) (3-5,20,21), micro electro-mechanical system (MEMS)-opticbased microfilter (3,22), ScreenCell® (23), CellSieve™ (24) and CellOptics® (3). Size-based enrichment techniques are jeopardized by the heterogeneity of size and the shape of CTCs. Filtration by size consents to enrich CTCs from a wide range of tumors, but sometimes results in loss of smaller CTCs or clotting of filter pores by leukocytes. Another morphology-based enrichment strategy is based on density gradient centrifugation using Ficoll-hypaque solution (6). Ficoll density-gradient dependent approaches are easy to handle, even if real losses of tumor cells have still been observed (25). Subsequently, the OncoQuick™ device was developed to avoid the cross-contamination of different layers by using a porous membrane, which keeps them separate (7). To date, several other devices based on physical properties of CTC are available, including a photoacoustic flow cytometer, a label-free biochip that exploits the differences in size and deformability of CTCs, a microfluidics device that combines multiorifice flow fractionation (MOFF) and the dielectrophoresis (DEP) cell separation techniques, and a DEP field-flow fractionation device that allows isolation of viable CTCs by their different response to DEP (26-30). Particularly, Gupta *et al*. have recently described the ApoStream device. This technique exploits differences in the biophysical characteristics between normal blood cells and cancer cells in order to capture CTCs using dielectrophoretic technology in a microfluidic flow chamber (31).

Most of CTC enrichment procedures involve immunomagnetic isolation (32-36). CTCs are subjected to positive or negative selection, utilizing either tumor cell antigens such as epithelial cell adhesion molecule (EpCAM), or hematopoietic cell antigens such as the common leukocyte antigen CD45 for purified cell suspensions (37-39). Antibodies are coupled to magnetic beads, thus the antigen–antibody complex is subsequently isolated from the solution with a magnetic field (8). Unfortunately, the lack of reliable target antigens for cellular capture still represents a significant limitation to the procedure. EpCAM, for example, is the by far most used capture antigen (i.e., CellSearch® system, CTC-Chip, MACS, Dynabeads, RosetteSep, affinity-based microchips) due to its expression across numerous tumor entities (40,41), but several pitfalls exist, including epithelial mesenchymal transition (EMT) and differential antigen expression. Particularly, EMT is a morphogenetic process in which cells lose their epithelial characteristics and acquire a mesenchymal-like migratory phenotype, endowing cells with invasive properties, thereby contributing to the apparition of CTCs and to the formation of metastases. It was further suggested that induction of EMT might generate cells that exhibit molecular and functional stem-like characteristics, leading to the under-expression of epithelial antigens like EpCAM (42). Thus, in order to capture this crucial biological subset of EpCAM low/negative CTCs, which have been suggested to confer aggressive tumor progression (43), future positive separation strategies should take this phenotype into account.

Recently, a structured medical Seldinger guidewire (FSMW), used to obtain safe access to blood vessels, bound with EpCAM antibodies, has been developed. This device has the potential to enrich CTCs *in vivo* and has been able to enrich EpCAM-positive CTCs from 22 of 24 BC or non-small cell lung cancer (NSCLC) patients (44). Finally, a novel technique using surface-enhanced Raman spectroscopy (SERS) has been described. This method is able to enumerate targeted CTCs in the presence of whole blood, using magnetic beads and SERS tags respectively conjugated to EpCAM and HER2 antibodies (45,46). SERS nanoparticles, with epidermal growth factor peptide as a target, successfully identified CTCs in the peripheral blood of 19 patients with squamous cell carcinoma of the head and neck (47).

More recently, novel methods combining physical (size) and biologic (immunomagnetic) features of CTCs have been developed. Particularly, the CTC-iChip is capable of sorting rare CTCs from whole blood at a rate of 10 million cells per second in both epithelial and non-epithelial cancers by using tumor antigen–independent microfluidic technology (48,49).

CTC detection

After enrichment, the solution usually still contains several leukocytes, thus CTCs need to be identified at the single-cell level and separated from normal blood cells. CTCs detection can be done through cytometric strategies or nucleic acid-based techniques (12).

Among cytometric strategies, classic immunocytochemistry (ICC) is the most widely used immunological approach, and has the advantage to facilitate classical cytopathological review. Furthermore, monoclonal antibodies against various

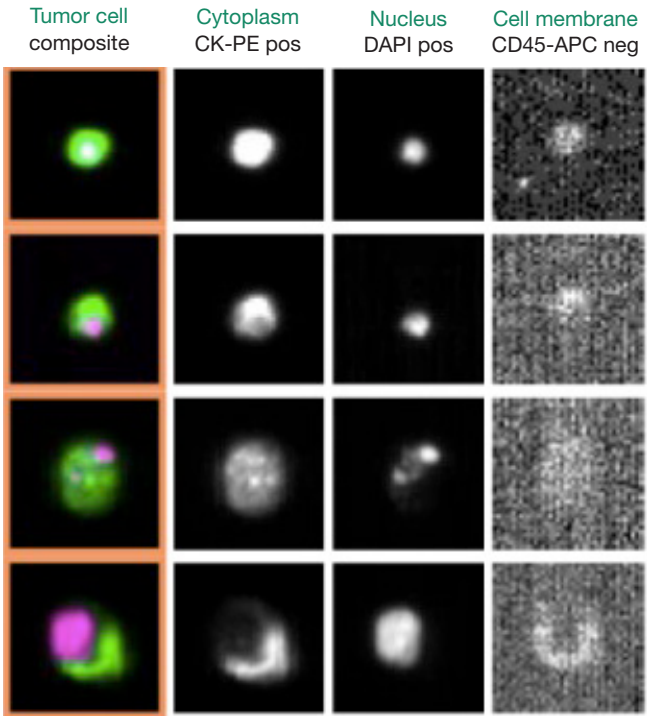

Figure 1 Computer-generated reconstruction of CTC images by semi-automated fluorescence-based microscopy system (CellSpotter Analyzer). EpCAM-positive cells are treated with a nucleic acid dye 4', 6-doamidino-2-phenylindole (DAPI), a leukocyte-specific anti-CD45 monoclonal antibody (CD45-APC) and epithelial-specific anti-cytokeratin 8, 18, and 19 antibodies (CK-PE). CTCs exhibit cytoplasmic expression of cytokeratin (second column), contain a nucleus that binds to the nucleic acid dye (third column) and they are CD45-negative (fourth column). The first column represents the composite of the cells. CTCs, circulating tumor cells; EpCAM, epithelial cell adhesion molecule.

epithelium-specific antigens, surface adhesion molecules, and growth factor receptors as well as diverse other upstream analyses (transcriptome/genome analyses) have been developed.

Among the current EpCAM-based technologies, the FDA cleared the CellSearch™ platform and the Ariol system (36), but the CellSearch™ remains the "gold standard" for all the CTC-detection strategies (8). The previously enriched EpCAM-positive cell fraction is additionally treated with a nucleic acid dye, a leukocyte-specific anti-CD45 monoclonal antibody and epithelial-specific anti-cytokeratin 8, 18, and 19 antibodies. Subsequently, a semi-automated fluorescence-based microscopy system (CellSpotter Analyzer) consents a computer-generated reconstruction of cellular images. CTCs express EpCAM and are CD45-negative, exhibit cytoplasmic expression of cytokeratin and contain a nucleus that binds to the nucleic acid dye 4', 6-doamidino-2-phenylindole (DAPI). The absence of one of these characteristics disqualifies a cell image as a CTC (*Figure 1*).

In 2007, Nagrath *et al.* introduced the "CTC-Chip", a microchip technology on a microfluidic platform that separates CTCs from whole blood using microposts coated with an antibody against EpCAM under precisely controlled laminar-flow conditions. In the pilot study, the CTC-chip successfully identified CTCs in the peripheral blood of 99% patients with metastatic lung, prostate, pancreatic, breast and colon cancer (10). In a first clinical and promising approach, the chip had been tested on the samples of NSCLC patients, demonstrating that changes in tumor genotypes (EGFR mutational analysis on DNA of CTCs) may correlate with response to treatments (50,51). More recently, Stott *et al.* improved the technique in patients with localized and metastatic prostate cancer. The authors associated the detection of the PSA with the EpCAM-based method and with morphologic criteria and integrated multiple signals in the same 3D microfluidic device, improving the efficacy of the CTC-chip to detect CTCs. The pilot study showed that CTCs rapidly decreased after surgical tumor removal or after the initiation of an effective treatment, while the persistence of CTCs after 3 months from the surgery suggested that CTCs could be released from the localized disease before metastases development (11). Other microfluidic chips used for the identification and isolation of CTCs include the IsoFlux system (based on immunomagnetic capture) (52,53) and the Herringbone-Chip (based on microvortices that increase the number of interactions between CTCs and the antibody) (54).

Nevertheless, EpCAM-based technologies do not consent to recognize whether the detected CTCs are viable or apoptotic cells. A new functional test that allows the detection of only viable cells after CD45-cell depletion has been developed for the CTC and DTC analyses. Avoiding direct contact with the target cells, this method assesses the presence of CTCs on the basis of proteins secreted or released during a 24-48 hours of short-term culture (i.e., CK19 and MUC1). This technique, named EPithelial ImmunoSPOT (EPISPOT) assay, has been applied to blood and bone marrow samples of breast, prostate and colon cancer patients providing first clinical data (13,14).

In 2011, Balic *et al.* developed a multi-marker imaging approach using DyLight technology (15). This technique requires the use of multiple antibodies (i.e., against

CK, HER2, ALDH1, CD44, and CD24) labeled with fluorochromes of different colors and spectral image analysis to separate different color spectra. Interestingly, by the addition of specific markers, this method may help to identify subpopulations that express particular therapeutic targets. Furthermore, the advent of quantum dots (QDs) with narrow emission spectra provided a new tool for multi-marker analysis. Compared to immunofluorescent dyes, QDs are brighter, not prone to photo bleaching, available in a number of colors, and their emission can be tuned to any desired wavelength by modulating the size of the particle (41,55,56). Other available immunofluorescence-based technologies for CTC-detection include automated scanning devices such as the fiber-optic array scanning technology (FAST) (57), the laser scanning cytometer [i.e., Maintrac® (58)] and a dedicated image cytometer [CellTracks® (59)].

Nucleic acid-based techniques have become the most widely used alternative to immunocytochemical assays. Particularly, PCR-based assay evaluates the amount of DNA from CTCs. The main disadvantage of this technique is the inability to distinguish the DNA free in the blood from apoptotic cells, creating false-positive results. For this reason, most groups prefer RT-PCR assays to target specific mRNA, since only viable CTCs produce mRNA (12,60,61). RT-PCR is based on the utilization of several cancer-related genes or epithelial antigens, including CK19, CK7, HER2, and mammaglobin A (62,63). To date, the mRNA encoding CK19 has been the most widely studied in clinical trials (64). Particularly, Stathopoulou *et al.* developed an RT-qPCR assay for KRT19 mRNA that showed to be highly sensitive and specific for the molecular detection of occult carcinoma cells in peripheral blood of BC patients (65-69). Nevertheless, the principal limitations to these techniques are related to the mRNA markers used, since they may be also present at low concentrations in normal blood, bone marrow cells and in other non-tumor cells (70). Moreover, cancer cells express high genetic instability and, especially in the course of the EMT, gene transcription may be downregulated. Quantitative real-time PCR provides interesting prospects for better quantification of the tumor cell load, provided that the specificity of the applied markers is well controlled.

AdnaTest (Alere) is a commercially available RNA-based CTC assay. This RT-PCR based assay utilizes nonquantitative RT-PCR to identify putative transcripts of genes after immunomagnetic separation of MUC1/HER2/EpCAM-positive cells (17). The principal limitation, along with the others related to EpCAM-based methods, is that MUC1 expression has been found on activated T lymphocytes (18). Another aspect to be considered is the fact that the RT-PCR is unable to quantify the tumor cell load, since the observed bands may be the result of one single cell as well as a thousand. Another promising nucleic acid-based technique is the RNAscope technology used by CTCscope. This assay, recently described by Payne *et al.* (16), measures single RNA molecules for the detection of single CTCs in metastatic BC patients. This is a method that requires minimal enrichment and that can exclude apoptotic cells, since these do not produce mRNA.

Concluding, in the last two decades several promising CTC detection methods have been developed. These strategies should be validated in appropriately sized clinical trials in order to evaluate their quality and validity.

The rationale for the use of CTCs

The presence of tumor cells in the peripheral circulation was reported for the first time in 1869 by Thomas Ashworth (71). Since then, the existence, origin, and clinical significance of CTCs have been widely discussed. In the late 1970s, the introduction of sensitive and specific immunohistochemical techniques led to renewed interest in the detection of CTCs and their possible association with early metastasization in solid malignancies. However, the lack of sensitivity of the early detection methods on circulating blood and the analogy between tissue metastasis and single cell precursors of solid metastasis, especially in bone, shifted the focus on the detection of DTCs in bone marrow. Bone marrow is accessible by needle aspiration through the iliac crest, and represents the most common homing organ for DTCs derived from different tumors and thus, the most prominent indicator organ for minimal residual disease (72,73).

Initially, DTCs have been detected in the bone marrow of 30-40% of primary BC patients and their presence has been strongly associated with poor prognosis (74). In more recent studies, the DTC detection rate appears to be far lower (about 3%), likely due to the earlier detection of BC ubsequent to increased use of screening mammography (75). Although the presence of DTCs is a significant prognostic factor in the prediction of outcome, the low rate of DTCs questioned the value of its routine use in this highly selective group of patients. Furthermore, bone marrow biopsy is an invasive procedure, with higher morbidity and costs than a simple blood draw, thus subsequent research was directed to

the evaluation of CTCs in peripheral blood. Nevertheless, it should be noticed that with the choice of focusing research on the blood compartment, epidemiologic, prospective and biologic evidence regarding CTCs must not simply be extrapolated from the extensive body of evidence on DTCs. Interestingly, several studies compared both compartments in the same patients and reported higher prevalence of DTCs in bone marrow than CTCs in the blood, and rates of CTC-DTC concordance ranging from 63% to 94% (76-82).

A higher presence of DTCs, together with its prognostic significance, favors the notion that bone marrow may be a 'perfect niche' in which tumor cells resist host defense mechanisms and survive. Several researchers shared the theory that bone marrow may represent a functional reservoir for cancer cells with the capability of recirculating through the bloodstream and colonizing other distant organs (83,84). As proof of that, a number of studies demonstrated that DTCs are able to persist in bone marrow even after completion of adjuvant therapy. Most of initially DTC-positive tumors turned negative during adjuvant treatment, but those with persistence of DTCs had worse disease-free and OS, suggesting that evaluation of DTCs can help in the selection of patients that will benefit from additional or a switch of adjuvant treatments (85). Even in neoadjuvant setting, the presence of DTCs in locally advanced BC was found to be a significant prognostic factor for cancer-related death, as well as a surrogate predictor of response to neoadjuvant chemotherapy and of disease recurrence (86). However, despite the presence of DTCs in bone marrow, according to the theory of "metastatic inefficiency" (87,88), only a part of tumor cells will be able to survive at the secondary sites and determine tumor mass, therefore only 40-60% of patients will eventually develop a relapse (74,89).

Clinical application of CTCs in BC

CTCs in metastatic BC

In 2004, the seminal work by Cristofanilli *et al.* demonstrated that CTC count detected using the CellSearch® was an independent prognostic factor for progression-free survival (PFS) and OS in metastatic BC. The cut-off of 5 CTCs/7.5 mL has been identified to classified patients with good or poor clinical outcome (9) and subsequent studies have confirmed the prognostic value of CTCs with the same cut-off (90-92). The data of the pivotal study resulted in FDA-approval of the CellSearch® for prognosis and monitoring of patients with MBC. Interestingly, several authors have then shown that monitoring CTC levels enable prediction of treatment efficacy (93,94). Particularly, Cristofanilli *et al.* demonstrated that detection of CTCs before initiation of first-line therapy is highly predictive of PFS and OS, even more than traditional imaging techniques (based on RECIST criteria) (95,96), and that detection of elevated CTCs at any time during treatment, since the first cycle of therapy, is an accurate indication of subsequent rapid disease progression and mortality (90). A recent pooled analysis of 1,944 patients across 17 European centers has confirmed the independent prognostic role of CTC level on PFS and OS in metastatic BC patients. Patients with a CTC count of 5/7.5 mL or higher at baseline were associated with decreased PFS (HR 1.92, P<0.0001) and OS (HR 2.78, P<0.0001) compared with patients with a CTC count of less than 5/7.5 mL. Moreover, 3-5 and 6-8 weeks after start of treatment, increased CTC counts were associated with decreased PFS and OS. The authors concluded that survival prediction was significantly improved by addition of CTC count to the clinic-pathological models, while the carcinoembryonic antigen (CEA) and cancer antigen 15-3 (Ca 15.3) levels at baseline and during treatment did not add significant information to the model (92).

Since changes in CTC levels proved to reflect treatment responses as early as after the first cycle of chemotherapy, several other studies evaluated the predictive potential of CTCs, monitoring their dynamic in peripheral blood during specific treatments. Particularly, Smerage *et al.* have recently published the final results of the SWOG S0500 trial. The aim of this trial was to determine whether switching chemotherapy after 21 days of first-line chemotherapy, in patients with persistent increase in CTCs, could improve their OS. This study showed that early changing to another therapy improved neither OS nor PFS. Nevertheless, the authors concluded that for this population, there would be a need for a wider participation in trials of novel therapeutic agents at the time of progression, rather than moving on to further lines of standard chemotherapy (97). Furthermore, the CirCe01 trial aims to investigate the value of early CTC count-based switch in chemotherapy regimen in third-line or later settings.

With regard to molecular subtypes, in hormone receptor positive BC, detection of higher levels of CTCs may guide the selection of patients who would more likely benefit from chemotherapy rather than endocrine treatment. On the other hand, CTCs seem to lose their prognostic value in

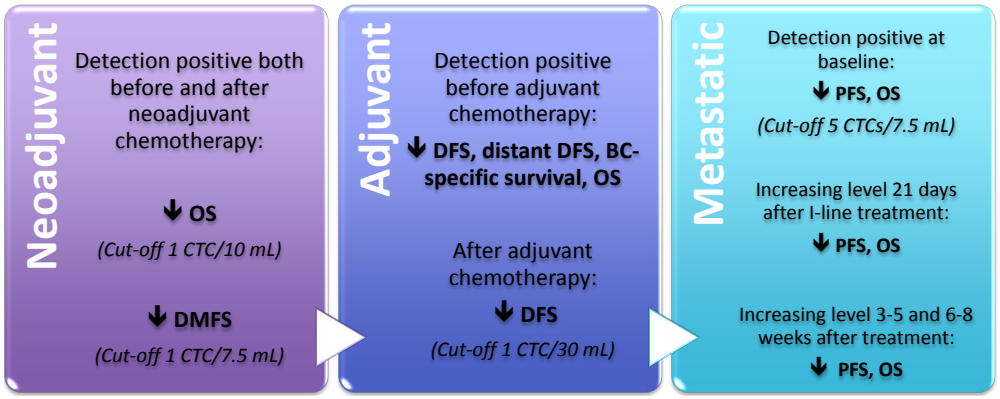

Figure 2 Prognostic and predictive value of CTC level in neoadjuvant, adjuvant and metastatic setting. OS, overall survival; DMFS, distant metastasis free survival; DFS, disease free survival; BC, breast cancer; PFS, progression-free survival; CTCs, circulating tumor cells.

patients with metastatic BC treated with targeted therapies, particularly in HER2 positive tumors. This effect could be due to a selective action of bevacizumab (Avastin®) and HER2-targeted therapy against circulating epithelial cells, reducing the prognostic value of CTCs enumeration (98-100).

Figure 2 reassumes the evidence for the prognostic and predictive value of CTC count in metastatic BC. CTCs enumeration could guide therapeutic decision-making and the development of tailored treatments, improving the management of metastatic patients.

CTCs in early-stage BC

In a large study involving 2,026 primary BC patients, CTCs have been detected in the peripheral blood of approximately 22% of patients after surgery and before adjuvant therapy (101). This amount appeared to be even higher in a smaller study that detected CTCs in approximately 31% of early stage BC patients (36% ER positive, 32% PR positive and 30% HER2 positive tumors). The authors reported that only 7% of all patients remained CTCs positive after adjuvant therapy, and no association between CTCs and tumor size, tumor grade, histological grade and receptor status was found (102). In adjuvant setting, CTC detection before chemotherapy has shown to be an independent predictor of disease-free survival (DFS) and OS and, not only the presence but also the quantity of CTCs has proven to be associated with worse outcome. Moreover, the persistence of CTCs after adjuvant treatment significantly correlates with a decreased DFS (101,103). These data have been recently updated and confirmed by Rack *et al.* in the success trial (104). In this large prospective trial, CTCs were detected in 21.5% of 2,026 patients before adjuvant chemotherapy. Particularly, CTCs were detected significantly more frequently in node-positive patients (22.4%) than in node-negative (19.6%) (P<0.001) while, no association was found with tumor size, grading, or hormone receptor status. Patients with at least 5 CTCs/30 mL blood before adjuvant treatment showed the worst prognosis. This trial provided strong evidence that CTC level represents a prognostic marker for reduced DFS, distant DFS, BC-specific survival, and OS before adjuvant chemotherapy and for DFS after completion of the treatment.

Therefore, CTCs evaluation in patients with early-stage BC could provide useful information for adjuvant treatment decision-making. However, in this particular context, CTCs are observed with low frequency thus, CTC detection methods with higher sensitivity could be necessary for their clinical use. Moreover, further studies are needed to better define its efficacy in both the prediction of outcome and monitoring the effect of therapy.

Concerning neoadjuvant chemotherapy, systemic response to treatment seems to be independent from the clinic-pathological features and the local response of the primary BC. Therefore, monitoring CTC and DTC levels during neoadjuvant treatment consents to better define the effectiveness of systemic treatment on tumor cell diffusion and could guide treatment strategies (105). In the neoadjuvant setting, CTCs have been detected in 22-23% of patients before and in 10-17% after systemic treatment. Interestingly, the persistence of CTCs after neoadjuvant chemotherapy was not correlated to the primary tumor response but it identifies a subpopulation of patients with an increased risk for early relapse and worse OS (106,107). *Figure 2* reassumes the evidence for the prognostic and predictive value of CTC count in neoadjuvant and adjuvant settings.

Clinical application of CTCs in other cancers

Prostate cancer

In 2005, Moreno *et al.* demonstrated that in patients with metastatic prostate cancer, CTC detection with the cut-off of 5 CTCs was a predictive factor superior to other clinical variables (108). Moreover, Okegawa *et al.* confirmed that CTC count represent an independent predictor for OS (109). Nevertheless, the optimal cut-off point to distinguish patients with favorable prognosis from those with poor prognosis still remains widely discussed (110-112). Subsequently, several large cohorts of castration-resistant prostate cancer (CRPC) patients clinically defined the prognostic significance of pre- and post-treatment CTC counts and the superior predictive ability of CTC enumeration in comparison with PSA level at all-time points (113-115). Therefore, changes in CTC counts in response to treatment have been established as indicators of response to treatment (116-118).

Patients with high-risk non-metastatic prostate cancer infrequently present with small number of CTCs in peripheral blood therefore, CTC may not be the optimal marker to predict prognosis or detect residual disease after radical prostatectomy (119,120). Interestingly, in these patients with localized prostate cancer, CTC count did not correlate with tumor volume, pathological stage, and Gleason score, suggesting that CTCs are more likely to originate from metastatic sites instead of primary lesions (121,122).

Colorectal cancer

In 2008, Cohen *et al.* demonstrated that metastatic colorectal cancer (mCRC) patients with ≥3 CTCs had significantly shorter median PFS and OS and worse treatment outcome compared with those with <3 CTCs (123). More recently, also the presence of at least 1 CTC at baseline count was found to be predictive for poor prognosis. Therefore, patients with 1-2 CTC should be switched from the favorable prognostic group (conventionally defined by the presence of <3 CTCs) to the unfavorable (124). Interestingly, the prevalence of CTCs in colorectal cancer patients are lower than in other cancer types, due to the capture of viable CTCs in the liver as first filter organ (125). Particularly, unfavorable baseline CTC was associated with worse PFS in patients receiving first- or second-line therapy, irinotecan, having liver involvement, ≥65 years, and ECOG PS of zero (126). Moreover, CTC count turned out to be a reliable surrogate biomarker in assessing Japanese patients responsive to oxaliplatin-based chemotherapy (127). More recently, high CTC count predicted reduced OS in patients treated with cetuximab-combination chemotherapy as third-line treatment (128).

In non-metastatic setting, molecular assessment for micrometastasis in sentinel lymph node along with CTC count may help to identify patients at high risk for recurrence and thus who could benefit from adjuvant therapy (129,130). Furthermore, even after curative resection, patients with persistence of CTCs exhibited higher incidence of relapse and worse relapse-free survival rate (131). In a multi-institutional study, a panel of genes (CEA/CK/CD133) investigated in peripheral blood from 753 colorectal cancer patients, turned out to be a superior prognostic factor over other existing clinicopathologic features in patients with Dukes' stages B and C (132). Particularly, higher CD133 expression was significantly associated with poorer clinical outcome and some clinicopathological factors such as T category, N category and vascular invasion in colorectal cancer patients (133).

Lung cancer

In lung cancer, it was demonstrated that CTC detection had the potential to distinguish malignant from benign lung disease and to predict the presence of distant metastasis. Moreover, CTC status was proportional to both clinical and pathological status and was associated with radiographic response at the end of two cycles of chemotherapy. CTC detection also possessed a significant prognostic value in both small and NSCLC patients who were treated with standard chemotherapy and also in resectable NSCLC independently of disease staging. Patients with a reduction in CTC number after one cycle of chemotherapy have longer PFS and OS (134-145).

Furthermore, the prognostic and predictive value of CTC level has been investigated in bladder, renal, ovarian, gastric and liver cancer (146). *Table 2* reassumes the main clinical evidence for CTC detection in breast, prostate, colorectal and lung cancer.

Liquid biopsy

As previously reported in literature, the immunehistochemical profile of BC could change in the course of the disease, determining a substantial discordance in receptor status

Table 2 The main clinical evidence for CTC detection in various types of cancer

Type of cancer	Tumor stage	Clinical results	References
Breast cancer (BC)	Metastatic	CTCs ≥5/7.5 mL associated with reduced PFS and OS	(9,92)
		CTCs C 5/7.5 mL at any time point during chemotherapy associated with adverse clinical outcome	(90)
		CTCs C 5/7.5 mL after the initiation of a new systemic therapy were associated with shorter median PFS and higher incidence of radiographic disease progression	(95,96)
		Lack of prediction in HER2-positive disease treated with targeted therapy	(99)
		Change in CTC level during the course of chemotherapy was a surrogate marker monitoring therapeutic efficacy and was correlated with OS	(147)
	Stages I-III	A low cutoff of 1 CTC was feasible. CTC detection was not associated with primary tumor response but was an independent prognostic factor for early relapse and OS after neoadjuvant therapy	(17,70,148,149)
		CTC detection incidence in early-stage disease paralleled with cancer stage. Each of CTC biomarker significantly correlated with ALN metastasis	(150)
		CTC detection before adjuvant chemotherapy was independent predictor of DFS and OS. The persistence of CTCs after adjuvant treatment significantly correlates with a decreased DFS	(66,68,103)
Prostate cancer	Metastatic	CTCs ≥5/7.5 mL was associated with poor OS	(108,151)
		Patients with unfavorable pre- or post-treatment CTC count had shorter OS and its predictive ability was superior to PSA level at all-time points	(152)
		CTC count predicted OS and was a sensitive marker in monitoring disease status during treatment, especially in early-stage disease	(114)
		CTC number as a continuous variable was prognostic for survival of patients with CRPC starting first-line chemotherapy	(115)
		CTCs ≥4/7.5 mL was associated with poor OS, and their presence correlates with radiographic findings and classic markers	(110)
		CTCs ≥2/7.5 mL was associated with disease status and clinical indicators of PSA and PSM	(111)
		CTCs ≥1.8/mL was associated with shorter OS	(112)
	Localized disease	No correlation between the number of CTC and known prognostic factor in localized prostate cancer patients	(121)
Colorectal cancer	Metastatic	The CTCs level at baseline and follow-up was an independent predictor of PFS and OS, which correlate well with radiographic findings	(123)
		The presence of at least 1 CTC at baseline count was found to be predictive for poor prognosis	(124)
		Unfavorable baseline CTC was associated with worse PFS in patients receiving first- or second-line therapy, irinotecan, having liver involvement, ≥65 years, and ECOG PS of zero	(126)
		CTC count was a reliable surrogate biomarker of predicting clinical outcome and assessing Japanese patients responsive to oxaliplatin-based chemotherapy	(127)
		CTC count during treatment could become a new predictor of therapy response	(128,130,153,154)
	Stage I-III	Patients with persistent presence of CTC even postoperatively exhibited higher incidence of postoperative relapse and poor relapse-free survival rate	(131)
		Molecular assessment for micrometastasis in sentinel lymph node along with CTC count may help to identify patients at high risk for recurrence and thus who could benefit from adjuvant therapy	(129,130)
Lung cancer	Localized and metastatic	The number of CTC was a robust surrogate prognostic indicator to predict the presence of distant metastasis. CTC status correlated with clinical and pathological stage	(134)
		CTC enumeration well correlated with radiographic response at the end of two cycles of chemotherapy	(135)
		CTCs decreased in all patients after on cycle of chemotherapy supporting CTC as a pharmacodynamic biomarker for small cell lung cancer (SCLC)	(137)
	Stages III-IV	CTC detection at baseline and change in CTC number after one cycle of chemotherapy was a prognostic factor for tumor response, PFS and OS	(136,138,140-144)
	Stages I-IV	CTC level ≥5 was associated with worse survival of patients with resectable NSCLC	(145)

CTCs, circulating tumor cells; PFS, progression-free; OS, overall survival; DFS, disease-free survival; CRPC, castration-resistant prostate cancer; NSCLC, non-small cell lung cancer.

between primary and recurrent BC (155). Particularly, the ER and HER2 status of the primary tumor could be discordant with the status of the metastatic tumor sites and the profile of CTCs (156). Since repeated and approachable tumor biopsies are invasive, costly and not always possible, the assessment of tumor characteristics on CTCs by a peripheral blood sample as a 'liquid biopsy' represents an attractive alternative.

Several studies analyzed the genetic aberrations carried by CTCs and compared their genetic profile to that of primary tumor, trying to correlate some mutations to disease aggressiveness and treatment response. Therefore, several clinical trials are currently investigating novel targeted strategies based on expression profiles of CTCs. For instance, Stebbing *et al.* studied the efficacy of lapatinib in metastatic BC with HER2-negative primary tumors and EGFR-positive CTCs, but the attempt to expand the pool of patients eligible for a targeted therapy in this study was unsuccessful (157). Moreover, the DETECT III trial and the CirCe T-DM1 trial are currently investigating the efficacy of HER2-targeted therapy in HER2-negative MBC with HER2-positive CTC.

Further interventional controlled phase III trials are needed to investigate and define the role of CTCs evaluation in the improvement of patient outcome and in the reduction of medical costs (158). It is likely that the future implementation of molecular and genomic characterization of CTCs will contribute to improve the treatment selection and thus to move toward precision medicine.

Conclusions

DTCs in bone marrow and CTCs in peripheral blood have a wide range of potential applications, including prognostication at diagnosis, assessment of treatment response, detection of early metastasization and evaluation of novel agents, allowing a personalized choice of treatment modalities and timing. Moreover, the capability of detecting and eradicating metastatic cells at an early phase of metastatic process likely has the potential to improve cancer outcomes. These interesting findings provide ample room for well-designed clinical trials in order to further investigate the significance of CTCs in human cancer.

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