

REVIEW

Minimal residual disease and circulating tumor cells in breast cancer

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Abstract

Tumor cell dissemination in bone marrow or other organs is thought to represent an important step in the metastatic process. The detection of bone marrow disseminated tumor cells is associated with worse outcome in early breast cancer. Moreover, the detection of peripheral blood circulating tumor cells is an adverse prognostic factor in metastatic breast cancer, and emerging data suggest that this is also true for early disease. Beyond enumeration, the characterization of these cells has the potential to improve risk assessment, treatment selection and monitoring, and the development of novel therapeutic agents, and to advance our understanding of the biology of metastasis.

Introduction

Breast cancer (BC) is the most common cancer in women in Europe [1]. Despite surgery and adjuvant systemic therapy, many women with early BC still relapse and die of their disease. Minimal residual disease (MRD) after potentially curative surgery for BC is thought to contribute to disease relapse and to be the target of adjuvant treatment. MRD is defined as micrometastatic cells undetectable by conventional imaging and laboratory tests. Surrogates of MRD are tumor cells detected in the bone marrow (disseminated tumor cells (DTCs)) and peripheral blood (circulating tumor cells (CTCs)) [2]. The detection and characterization of DTCs/CTCs are expected to lead to personalized treatment strategies and accelerate the development of novel therapeutic agents for BC [2]. Furthermore, genotypic and phenotypic characterization of DTCs/CTCs at the single cell level

may provide novel insights into the biology of tumor progression [3].

Detection methods

The detection of DTCs/CTCs in BC is challenging since these cells are rare, occurring at a frequency of one tumor cell per 10^6 to 10^7 mononuclear cells. To isolate DTCs/CTCs, enrichment techniques are therefore typically applied. These techniques are based either on the physical properties of the cells (for example, cell density by ficoll centrifugation or cell size by filtration) or on their immunological characteristics (for example, cell surface antigens of DTCs/CTCs by immunoenrichment or markers of hematopoietic cells by immunodepletion). Ficoll centrifugation was widely used in the initial clinical studies of bone marrow DTCs [4]. Currently, however, enrichment techniques incorporating immunomagnetically labeled monoclonal antibodies are more often used because they improve tumor cell recovery (recovery rates of >50% to 85%) [5,6] over ficoll enrichment (recovery rate of 40%) [7] in spiking experiments using cell lines. After the initial enrichment step, DTCs/CTCs have been detected using assays based on either antibodies (immunocytochemistry, immunofluorescence) or nucleic acids (mRNA transcripts by reverse transcription PCR (RT-PCR)). Table 1 summarizes the main technologies for CTC detection in breast cancer.

Antibody-based assays

Since there are no universal tumor-specific antigen/genes, epithelial-specific antigens, including cytokeratins (CKs), epithelial cell adhesion molecule (EpCAM), and growth factor receptors (for example, human epidermal growth factor receptor (HER2)), have been used as markers of choice for the detection of DTCs/CTCs. In consensus meetings, the use of appropriate staining controls, directly labeled fluorescent monoclonal antibodies, identification of DTCs/CTCs based on cytomorphologic criteria/phenotypic features, and validation by two independent observers have been suggested as measures to reduce false-positive results [8,9].

CellSearch[®] (Veridex, Warren, NJ, USA) is an automated enrichment and immunostaining system for CTC

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Table 1. Technologies for circulating tumor cell detection in breast cancer

System	Enrichment	Detection	FDA approval	Reference
CellSearch®	EpCAM-coated ferrofluids	CK8, 18, 19+/DAPI+/CD45-	Yes*	[10-12]
CTC-chip®	Microfluidics. EpCAM-coated microposts	CK+/CD45-/DAPI+	No	[29,34]
The CTCChip®	Microfluidics. Enrichment based on physical properties	CK+	No	[31]
Magsweeper®	EpCAM+ enrichment	Gene expression profiling	No	[6]
EPISPOT assay®	Depletion of CD45+ cells	CK19, MUC1	No	[35]
MAINTRAC®	RBC lysis	EpCAM+/CD45-	No	[16]
Ariol®	RBC lysis, CK+, EpCAM+ enrichment	CK8, 18, 19+/DAPI+/CD45-	No	[19]
Fiber-optic array scanning technology (FAST)	No	CK+	No	[20]
Collagen adhesion matrix (CAM) assay	Invasion and digestion of cell adhesion	EpCAM+, panCK+/CD45-	No	[36]
AdnaTest®	MUC1+ and EpCAM+ enrichment	HER2, MUC1 and EpCAM	No	[28]
Single gene or multi-marker RT-PCR assays	Ficoll or EpCAM+ enrichment or depletion of CD45+ cells	CK19, MGB1, HER2, MUC1 mRNA	No	[22-27]
Multiparameter flow cytometry	Ficoll enrichment	EpCAM+/CK8, 18, 19+/CD45-	No	[15]

CK, cytokeratin; EpCAM, epithelial cell adhesion molecule; FDA, Food and Drug Administration; HER, human epidermal growth factor receptor; MGB1, mammaglobin-A; MUC1, mucin 1; RBC, red blood cell. *Approval as an aid in monitoring patients with metastatic breast, colorectal and prostate cancer.

detection that uses microscopic ferrofluids coated with an antibody against EpCAM to magnetically separate epithelial cells from whole blood [10]. Captured cells are stained with antibodies specific for cytokeratins 8, 18 and 19 (pan-CK) and CD45 (specific for leucocytes) and stained with 4'6-diamidino-2-phenylindole-2 (DAPI; to confirm the presence of a cell nucleus). A CTC is defined as a cell staining for pan-CK and DAPI, but not for CD45. Currently, CellSearch® is the only technology that has received US Food and Drug Administration (FDA) approval for CTC detection as an aid in monitoring patients with metastatic breast, colorectal and prostate cancer [10-12]. The performance of CellSearch® for CTC detection in metastatic solid tumors has also been validated in ring studies [13,14].

Other technologies include the MagSweeper, which uses immunomagnetic separation and gently enriches target cells by 10⁸-fold from blood, eliminating cells that are not bound to magnetic particles [6]. This process has been shown to keep cell function intact and not to perturb rare cell gene expression [6]. CTCs have been also detected using multi-parameter flow cytometry, and their detection with this technology was associated with poor outcome in women with BC [15]. MAINTRAC®, another method, detects circulating epithelial tumor cells from whole unseparated blood, and uses a laser scanning cytometer after staining with anti-human epithelial and anti-CD45 fluorescent antibodies [16]. This technology results in CTC counts up to 10⁵ per milliliter of blood in all women with early BC, consequently raising concerns about the specificity of the method to detect tumor cells [16].

Advances in optical technologies have also improved DTC/CTC detection. Several slide-based automated microscopic scanning devices, such as the Ikoniscope® [17] and the Ariol® system [18,19], have been applied for standardized micrometastatic cell detection and characterization. Another approach has been developed that uses fiber-optic array scanning technology (FAST) for rare cell detection [20]. It has been demonstrated that FAST cytometry is capable of a 500-fold increase in speed over automated digital microscopy, with comparable sensitivity and superior specificity [20]. The combination of FAST and automated digital microscopy has allowed investigators to detect rare epithelial cells from whole unseparated blood after immunofluorescence staining with a pan-CK antibody.

Nucleic acid-based assays

Nucleic acid-based assays have been initially hampered by false-positive results due to inability to assess tumor cell morphology, expression of target genes in normal cells, and the presence of pseudogenes (genes without protein-coding abilities) [21]. Newer quantitative assays have addressed some of these problems. To detect DTCs/CTCs in breast cancer, nucleic acid-based assays, either as single genes or as part of multiplex assays [22-27], have mainly used *CK19*, mammaglobin-A (*MGB1*), *HER2* and mucin 1 (*MUC1*) mRNA. The AdnaTest® BreastCancerSelect (AdnaGen AG, Langenhagen, Germany) is a commercially available molecular assay that utilizes immunomagnetic separation with antibodies against MUC1 and EpCAM followed by a multiplex RT-PCR for *HER2*, *MUC1* and *EpCAM* [28].

Emerging detection technologies

Beyond immunomagnetic separators, microfluidic devices have been developed for rare cell tumor capture, and these involve non-electrokinetic methods, such as immobilization via antibody [29] and size-based sorting [30,31], or electrokinetic methods (for example, dielectrophoresis) [32]. An example of a microfluidic platform is the 'CTC-chip', which is capable of efficient and selective separation of viable CTCs from peripheral whole blood samples, mediated by the interaction of target CTCs with EpCAM-coated microposts under precisely controlled laminar flow conditions [29]. A direct comparison between CellSearch® and two commercially available CTC-chips showed that these platforms provided similar sensitivity and yield in patient samples [33]. Stott and colleagues [34] recently reported improved sensitivity of the CTC-chip for CTC detection in patients with localized prostate cancer.

Several other assays have also been developed. For example, a technique named EPISPOT (epithelial immunospot) allows detection of viable DTCs and CTCs owing to their ability to secrete individual proteins after 48 hours of short-term culture [35]. A functional cell separation method called CAM, or the collagen adhesion matrix assay, was reported to detect CTCs with the invasive phenotype and to explore their molecular features [36]. Beyond these assays, new imaging procedures have been developed for the *in vivo* detection of CTCs [37].

Several investigators have also evaluated the potential utility of circulating cell-free DNA, either as a surrogate to monitor MRD [38], or as a 'liquid biopsy' for real-time monitoring of tumor mutations in cancer patients [39]. Moreover, some investigators have been able to identify patient-specific genomic rearrangements in plasma-circulating DNA as a way to monitor MRD [40]. They employed next-generation sequencing to rapidly identify patient-specific genomic rearrangements in primary tumors and showed that PCR assays could reliably detect these rearrangements in plasma [40]. A recent review has summarized advances in cell-free nucleic acids (DNA, mRNA, microRNA) as potential biomarkers in cancer [41].

Critical interpretation of detection technologies

The different technologies use different enrichment and detection steps and therefore do not always detect the same CTC population (Table 1). In a study comparing two commercially available assays (CellSearch® and AdnaTest®) in the same metastatic BC patient samples, the concordance between the two assays was 64% for CTC detection and 50% for HER2-positive CTC detection [42]. Therefore, it is important to study the clinical utility of the assay-dependent CTC detection and characterization. Moreover, most enrichment methods used by

the different assays are biased because they result in loss of a fraction of CTCs due to tumor cell heterogeneity. As an example, some available technologies detect only EpCAM+ CTCs (Table 1). However, it has been shown that BC cell lines with low EpCAM expression and high expression of mesenchymal markers cannot be efficiently captured using a purely EpCAM-based mechanism [33,43,44]. Some other technologies are using enrichment based on red blood cell lysis or leukocyte depletion (CD45-negative depletion) aiming at a less biased CTC enrichment (Table 1). Another critical issue with all cell detection technologies is that blood cannot be stored and must be processed soon after it has been drawn, within up to 72 hours [13] depending on the technology used. Therefore, the clinical validation of CTCs depends on the availability of detection technologies in different labs. This is a major difference between CTCs and biomarkers from paraffin-embedded primary tumor blocks, for which real-time processing is not mandatory. Since all currently available platforms will continue to evolve rapidly, the challenge will be to prospectively evaluate the utility of each technology to address specific clinical questions.

Clinical relevance of DTCs/CTCs

CTCs and DTCs were cited for the first time in the 2007 recommendations of the American Society of Clinical Oncology (ASCO) on tumor markers [45]. Recently, in the 7th edition of the American Joint Committee on Cancer Staging Manual (2010), a new M0(i+) category was proposed for TNM (tumor, node, metastasis) staging in BC [46]. This new category is defined as no clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumor cells (no larger than 0.2 mm) in blood, bone marrow, or other non-regional nodal tissue in a patient without symptoms or signs of metastases.

Clinical relevance of DTCs

The inclusion of the M0(i+) category was driven at least in part by a pooled analysis of individual data from several studies, which showed that bone marrow CK-positive DTCs were detected at the time of surgery in 30.6% of 4,703 patients with invasive BC [4]. Bone marrow DTCs were significantly more frequent in women with larger tumors, or tumors with higher histologic grade, hormone receptor negativity, and lymph node metastasis. In multivariate analysis, the presence of bone marrow DTCs predicted for significantly higher risk of death from BC [4]. Recently, in the American College of Surgeons Oncology Group's (ACOSOG) Z0010 multicenter trial, bone marrow DTCs were identified at surgery by immunocytochemistry in only 105 of 3,491 patients (3%) with clinical T1/T2 N0 M0 BC [47].

Although the DTC detection rate was very low, bone marrow DTCs still significantly predicted decreased overall survival [47]. A pooled analysis of individual patient data from 676 women with stage I-III BC from three studies showed that bone marrow DTCs were detected in 15.5% of patients at a median 37-month follow-up after diagnosis [48]. The presence of DTCs was an independent indicator of poor prognosis and could be used to select patients for secondary adjuvant treatment strategies [48].

Clinical relevance of CTCs (CellSearch®)

Using CellSearch®, ≥ 5 CTCs/7.5 ml of blood were detected in 49% of 177 patients with measurable metastatic BC before a new treatment was started [10]. CTC detection was an independent predictor of progression-free survival and overall survival [10]. This and other studies [49-52] have provided solid data about the adverse prognostic value when CTCs are detected by CellSearch® in metastatic BC.

Detecting CTCs in non-metastatic BC is more challenging because these cells occur at a very low frequency in this setting. Pierga and colleagues [53] found ≥ 1 CTC/7.5 ml in 23% of 97 patients before administering neoadjuvant chemotherapy (NAC) and in 17% of 86 patients after NAC. The detection of ≥ 1 CTC/7.5 ml before NAC, after NAC, or at both time points in the above study was associated with worse distant metastasis-free survival and overall survival at a median follow-up of 36 months [54]. In another study ≥ 1 CTC/7.5 ml were detected in 21.6% of 213 patients before NAC and in 10.6% of 207 patients after NAC [55]. In both these studies, however, neither CTC detection before or after NAC, nor changes in CTC detection during treatment, were predictive of pathological complete response [53,55]. Rack and colleagues [56] detected ≥ 1 CTC/22.5 ml before the start of adjuvant treatment in 21.5% of 2,026 patients with early BC [56]. In this study, pre-treatment detection of CTCs was confirmed as an independent predictor for both disease-free survival and overall survival [56]. Several other investigators have detected CTCs by CellSearch® in 9% to 38% of patients with early BC without reporting survival data [57-59].

These differences in CTC detection rate in early BC could be attributed to the Poisson distribution of rare events [60], to differences in patient populations, sampling time points, blood volume analyzed, the use or not of ficoll enrichment before processing with CellSearch®, and differences in image interpretation between different labs. Most women in this setting have only one detectable CTC/whole blood volume analyzed. Therefore, in order to prospectively test potential clinical applications of CTCs in non-metastatic BC, it is important to standardize image interpretation across labs by taking into account cytomorphologic criteria.

CTCs versus DTCs

Since blood is more easily obtained than bone marrow, an important question is whether peripheral blood CTCs can be used as surrogate markers for bone marrow DTCs. In one study, peripheral blood and bone marrow were collected from 341 patients at a median follow-up of 40 months after initial surgery [61]. In this study, 8 patients were CTC+/DTC+, 26 were CTC+/DTC-, and 40 were CTC-/DTC+. Although both CTCs (10% of the patients) and DTCs (14% of the patients) were significantly associated with worse clinical outcome, DTCs were more informative than CTCs [61]. This and other studies [62] showed that there was no good correlation between CTC and DTC detection. However, it is not clear whether this is because CTCs and DTCs represent different tumor cell populations or whether this is also related to limitations of the detection technologies used. At present there are no data to support that CTCs can replace DTCs.

Clinical relevance of nucleic acid-based assays

In early BC, initial single-center studies have reported that the detection of peripheral blood *CK19* mRNA by RT-PCR after ficoll enrichment of mononuclear cells was an independent prognostic factor for reduced disease-free survival and overall survival [63,64]. In another study, 13% of 431 early BC patients were CTC-positive according to the AdnaTest®; however, no correlation with clinical outcome was reported [65]. In metastatic BC, CTC detection by AdnaTest® was reported in 52% of 42 women and predicted therapy response in 78% of cases [66]. Finally using immunomagnetic tumor cell enrichment and a multi-marker quantitative PCR based assay, CTCs were detected in 7.9% of 733 stage I/II breast cancer patients with a median follow-up time of 7.6 years and their detection was an independent predictor of metastasis-free survival and breast cancer specific survival [67]. However, despite these initial results, no nucleic acid-based assay has received FDA approval nor has demonstrated utility in treating patients with BC.

Clinical trials with DTCs/CTCs

Interestingly, CTC or DTC clearance after systemic treatment has been used as an endpoint in BC clinical trials. In one single-center study, it was shown that a short course of trastuzumab (3 cycles every 3 weeks) eliminated chemotherapy-resistant *CK19* mRNA-positive cells in peripheral blood or bone marrow in 20 of 30 women with stage I-IV BC [68]. Another study randomized women with stage II and III BC to NAC with or without zoledronic acid [69]. The primary endpoint of the trial was the number of patients with detectable DTCs at 3 months' post-treatment. At 3 months, DTCs were detected in 17 of 56 patients receiving zoledronic acid

versus 25 of 53 patients who did not. Although fewer women had detectable DTCs after NAC with concurrent zoledronic acid than with chemotherapy alone, this was not the case when only women who tested DTC-positive at baseline were analyzed. A critical question is if CTC clearance can be used as a 'surrogate' for survival for regulatory purposes. Such an effort is ongoing and investigators are studying CTC detection by CellSearch® before and after treatment in the phase 3 registration trials of abiraterone acetate in prostate cancer [70].

Although data on the adverse prognostic value of CTC detection by CellSearch® in metastatic BC are solid, evidence from prospective trials is needed that CTC detection can lead to changes in treatment decision and thus improve clinical outcome in metastatic BC. Such an effort is ongoing in a phase III trial run by the Southwest Oncology Group, which is testing the strategy of changing chemotherapy versus continuing the same chemotherapy for patients with metastatic BC who have elevated CTC levels at their first follow-up assessment (ClinicalTrials.gov NCT00382018).

DTC/CTC characterization

Identification of therapeutic targets

Beyond enumeration, further characterization of DTCs and CTCs holds the promise to improve treatment outcome in women with BC. Because of the availability of anti-HER2 agents, HER2 expression was studied on DTCs [71-73] and CTCs [33,42,55,59,66,74-78] (Table 2) and was correlated with HER2 expression on the primary tumor. In most studies, HER2 expression on DTCs/CTCs is more prevalent in women with HER2-positive BC than in women with HER2-negative BC in both non-metastatic and metastatic settings. Interestingly, among women with HER2-negative primary tumors defined by standard pathology and detectable CTCs, between 14% and 50% may have at least one HER2-positive CTC. However, it is not known whether the discordant cases can be attributed to technical causes or whether there is any underlying biological explanation. Clinical testing for HER2 in the primary tumor is known to result in false-negative and false-positive results [79]. Furthermore, in most cases different technologies are used to evaluate HER2 in the primary tumor and the CTCs. Beyond technical issues, functional HER2 protein up-regulation on CTCs cannot be excluded, and the acquisition of HER2 amplification during the course of the disease has been suggested [74]. It was shown that four out of nine patients with metastatic BC whose primary tumors were HER2-negative and who had CTCs showing *HER2* gene amplification derived benefit from trastuzumab-containing therapy [74].

Beyond HER2, several other markers have been studied on DTCs/CTCs. Markers related to angiogenesis, such as

vascular endothelial growth factor (VEGF), VEGF2, and hypoxia inducible factor (HIF)-1 α , were observed in CTC-positive samples from metastatic BC patients [80]. Using the CTC-chip technology to purify CTCs, epidermal growth factor receptor mutations conferring drug resistance were detected in CTCs from non-small-cell lung cancer patients who had received tyrosine kinase inhibitors [81]. Androgen receptor mutations were also identified in CTC-enriched peripheral blood samples from castration-resistant prostate cancer patients [82]. In most of these studies, DTC/CTC characterization was performed in few patients in the metastatic setting, and therefore validation in independent larger patient series is required. Characterizing DTCs/CTCs in non-metastatic tumors poses additional challenges since such cells are only rarely detected in this setting. Finally, clinical trials are needed to demonstrate that CTC characterization is important for patient management.

Identification of DTCs/CTCs with 'tumor-initiating cell' phenotype

Beyond the potential for improving patient outcome, the study of DTCs/CTCs aims to lead to a better understanding of the metastatic process. Research has shown a significant proportion of DTCs to be resistant to conventional chemotherapy [48]. Furthermore, using Ki67 immunostaining, most micrometastatic cells have been found to be in a non-proliferative state [83]. Interestingly, the CD44⁺CD24^{-/low} tumor-initiating cell phenotype [84] was observed in a significant number of bone marrow DTCs using triple-staining by immunocytochemistry [85]. Moreover, the CK19⁺/MUC1 stem cell-like phenotype was demonstrated in a significant number of DTCs in BC by the EPISPOT assay [35]. Epithelial mesenchymal transition markers and aldehyde dehydrogenase 1 (ALDH1) were also identified in a major proportion of CTCs from patients with metastatic BC [86]. However, clinical studies are needed to associate the presence of CTCs/DTCs with tumor-initiating cell phenotype with clinical outcome in women with BC.

Tumor dormancy

An issue related to the role of DTCs in the metastatic process is determining which of them will grow into overt metastases and which will not. According to clinical studies on bone marrow DTCs, 50% to 70% of patients with detectable DTCs will not develop metastases, although even patients without DTCs may relapse and die of BC [4]. For patients who relapse without such cells detectable in their bone marrow, it is possible that the DTCs have actually settled into other organs; alternatively, lack of DTCs could be the result of sampling error or reflect the suboptimal sensitivity of CKs as a marker for DTC detection. Indeed, tumor cell dissemination has

Table 2. HER2 expression on circulating tumor cells and primary breast cancer

Stage	HER2 PT	Enrichment/detection; CTC positive; CTC HER2 positive	CTC detection rate	HER2+ CTCs/total CTCs (%)		Reference
				HER2- PT	HER2+ PT	
M0 and M1	FISH	EpCAM+ enrichment, C11, anti-CD45 (Her 81) HER2 CTC: IF, 0, 1+, 2+, 3+, FISH HER2+ CTCs: IF (3+), FISH HER2/CEP17 ≥ 2.0	NA	9/24 (37%)	11 of 15 (73%)	[74]
M0	IHC, FISH	Ficoll, immunomagnetic separation ICC CK, HER2 (21N clone) ≥ 1 HER2+ CTC/50 ml	17/35 (49%)	12/24 (50%)	2/3 (67%)	[75]
M0	IHC, FISH	CellSearch® ≥ 1 CTC/7.5 ml ≥ 1 HER2 (3+) CTC/7.5 ml (HER2 IF: 0, 1+, 2+, 3+)	Before NAC: 46/213 (22%) After NAC: 22/107 (11%)	8/37 (21%)	6/21 (28%)	[55]
IS and M0	IHC, FISH	CellSearch® ≥ 1 CTC/22.5 ml ≥ 1 HER2+ CTC/22.5 ml HER2 Intensity CellSearch® ≥ 2.5	IS: 6/73 (8%) M0:12/101(12%)	5/12 (41%)	5/5 (100%)	[59]
M0 and M1	IHC, FISH, CISH	CellSearch® ≥ 2 CTCs/7.5ml $\geq 50\%$ HER2+ CTCs (HER2 IF: -, +)	40/66 (61%)	8/28 (29%)	7/12 (58%)	[76]
M1	IHC, FISH	AdnaTest®, RT-PCR	22/42 (52%)	5/17 (29%)	2/5 (40%)	[66]
M1	IHC, FISH	AdnaTest®, RT-PCR CellSearch® ≥ 5 CTCs/7.5 ml ≥ 1 HER2 (3+) CTC/7.5 ml (HER2 IF: 0, 1+, 2+, 3+)	90/229 (30%) 122/245 (50%)	28/57 (49%) 25/76 (33%)	9/22 (41%) 18/31 (58%)	[42]
M1	IHC, FISH,	CellSearch® ≥ 1 CTC/7.5 ml ≥ 1 HER2+ CTC/7.5 ml (HER2 IF: -, +)	57/76 (75%)	6/42 (14%)	13/15 (87%)	[77]
M1	IHC, FISH	CellSearch® Profiling Kit, FISH for HER2 ≥ 1 CTC/7.5 ml HER2+ CTCs: FISH HER2/CEP17 ≥ 2.0	75/75 (100%)	10/30 (33%)	44/45 (98%)	[78]
M1	IHC, FISH	CellSearch® ≥ 1 CTC/7.5 ml HER2 IF: 0, 1+, 2+, 3+ H-score ≥ 200 for HER2 IF	29/38 (76%)	3/18 (16%)	9/11 (82%)	[33]

CISH, chromogenic *in situ* hybridization; CTC, circulating tumor cell; EpCAM, epithelial cell adhesion molecule; FISH, fluorescent *in situ* hybridization; ICC, immunocytochemistry; IF, immunofluorescence; IHC, immunohistochemistry; IS, ductal/lobular carcinoma *in situ*; M0, non-metastatic; M1, metastatic; NA, not available; NAC, neoadjuvant chemotherapy; PT, primary tumor; RT-PCR, reverse transcription PCR.

been linked to epithelial mesenchymal transition and the down-regulation of epithelial cell markers [87]. Conversely, it is possible that non-relapse in the case of patients with DTCs/CTCs can be attributed to the detection of apoptotic cells or to tumor dormancy. Interestingly, CTCs have been detected in one-third of women without clinical evidence of disease up to 22 years after mastectomy for BC [88].

There is evidence that several mechanisms of dormancy exist, including cellular dormancy, in which DTCs enter a state of quiescence (G0-G1 arrest), and tumor mass dormancy, in which DTCs divide but the lesion does not grow beyond a certain size [89]. There is also evidence that mechanisms regulating the switch between cellular

dormancy and escape from it are related to the cross-talk between DTCs and the microenvironment [89,90]. For example, loss or absence of a surface receptor like HER2, urokinase-type plasminogen activator receptor (uPAR) or integrins that transduce growth signals from the microenvironment may result in a dormant DTC, whereas the presence of such a receptor and a permissive microenvironment may result in a proliferating DTC. Interestingly, the overexpression of HER2 [71] or uPAR [91] on DTCs was associated with poor prognosis in patients with breast and gastric cancer, respectively.

The mechanisms that regulate the switch between tumor mass dormancy and expansion have been suggested to be related to angiogenesis [92] and the immune

system [93]. When there are limitations in blood supply and/or when there is an active immune system, for example, the micrometastasis cannot grow into overt metastasis. By contrast, a shift in favor of pro-angiogenic factors and activation of transcriptional programs that allow the recruitment of new blood vessels (angiogenic switch) or an escape of immune surveillance (immunosuppression) may cause the expansion of the micrometastatic cells into macrometastasis. It is not clear how all these mechanisms operate in a given patient, or how they are influenced by exogenous factors like stress and diet or by host genetic factors [94].

Genomic characterization of DTCs/CTCs

Thus far, only limited information is available about the global gene expression programs that determine the fate of DTCs and CTCs. Some studies have performed molecular characterization of CTC-enriched samples and reported mRNA or microRNA expression of CTC-specific genes in metastatic BC [95,96]. Using single cell comparative genomic hybridization, it has been shown that bone marrow DTCs are genetically heterogeneous and display fewer genetic aberrations than primary tumor cells [97-99]. In addition, the most prevalent chromosomal aberrations of primary breast tumors (including 8q gain, 13q loss, 16q loss and 17p loss) have rarely been found in DTCs with abnormal karyograms isolated at the time of curative surgery [3]. Husemann and colleagues [100] provided evidence that systemic spread occurs early in BC by showing that tumor cells can disseminate from earliest breast epithelial alterations in transgenic mice and from breast ductal carcinoma *in situ* in women. These results have led to the proposal of a parallel progression model in which tumor cells disseminate early at ectopic sites and evolve in parallel with tumor cells in the primary site [3]. Finally, beyond gene expression profiling and comparative genomic hybridization, the characterization of DTCs/CTCs using next generation sequencing may provide new insights into the cellular programs that regulate tumor dormancy and metastasis.

Future directions

The characterization of DTCs/CTCs might lead to the identification of targets for the design of new drugs. CTCs might also be used to accelerate drug development if ongoing or future trials demonstrate that CTC clearance is a 'surrogate' for drug efficacy. In order to move DTCs/CTCs into clinical practice, prospective trials with innovative designs and endpoints are needed to demonstrate both clinical utility and cost-effectiveness. Such efforts are currently ongoing. Because the technologies used to detect and characterize tumor cells in peripheral blood are rapidly evolving, issues like easy

access to newer technologies and standardization across laboratories will be critical for prospective validation. CTC detection and characterization have the potential to improve risk assessment and provide a 'liquid biopsy' for real-time monitoring of tumor genotype/phenotype in metastatic BC. In early BC, the presence of MRD after patients have completed standard adjuvant treatment may contribute to a better selection of patients to evaluate secondary adjuvant treatment strategies. Overall, the integration of information from both the primary tumor and MRD may eventually lead to personalized treatment strategies.

Abbreviations

BC, breast cancer; CK, cytokeratin; CTC, circulating tumor cell; DAPI, 4'-diamidino-2-phenylindole-2; DTC, disseminated tumor cell; EpCAM, epithelial cell adhesion molecule; FAST, fiber-optic array scanning technology; FDA, Food and Drug Administration; HER, human epidermal growth factor receptor; MRD, minimal residual disease; NAC, neoadjuvant chemotherapy; PCR, polymerase chain reaction; RT, reverse transcription; uPAR, urokinase-type plasminogen activator receptor.

Competing interests

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