

Review Article

Disseminated and Circulating Tumour Cells and Their Role in Breast Cancer

(breast cancer / detection / disseminated tumour cells / circulating tumour cells / bone marrow / metastasis / epithelial / mesenchymal transition / cancer stem cells / dormancy / HER2 / prognostic factor)

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Abstract. Metastatic spread of the primary tumour is responsible for the vast majority of cancer-related deaths. Detection of disseminated tumour cells in the bone marrow and circulating tumour cells in the peripheral blood is correlated with early metastatic relapse in breast cancer. Positive detection of disseminated tumour cells was associated with poor overall survival of patients. Current research has been focused on integrating minimal residual disease as a prognostic and predictive tool in the management of breast cancer. Detection of disseminated tumour cells/circulating tumour cells is not yet standardized in clinical practice because of using different enrichment and detection methods. Therefore, standardization of the used methods is necessary in the future. Previous achieved findings must be verified in larger prospective multicentre studies. Further characterization of disseminated tumour cells/circulating tu-

mour cells will be essential for developing and monitoring the efficacy of new therapeutic concepts. The aim of this review was to provide a short survey of the metastatic cascade and cancer stem cell theory, and data on the molecular and functional characterization of disseminated tumour cells/circulating tumour cells. Finally, we discuss the potential clinical impact of disseminated tumour cells/circulating tumour cells and results of several recent studies.

Introduction

Breast cancer is the most frequent cancer in women. It constitutes almost 20 % of all malignancies in women. Its incidence is high and constantly increasing. Currently it affects approximately 6 % of the female population. Despite recent advances in early diagnostics and treatment strategies, breast cancer is still a leading cause of cancer-related death among women, with as many as 40 % relapsing with metastatic disease (Rosen et al., 1989). Clinically detectable metastases are seen in 5 % of patients at the time of primary diagnosis and additional 30–40 % present occult metastasis (Clare et al., 1997; Braun et al., 2005) that will probably result in the disease relapse.

The relapse of cancer is caused by many factors. Determinant of outcome leading to the treatment modification is the absence or presence of metastatic dissemination of tumour cells at the time of initial presentation and during treatment (Pantel et al., 2009). Ultrasensitive approaches now enable detection of the tumour cells spread in the bone marrow (BM) called “disseminated tumour cells” or in the peripheral blood (PB) referred to as “circulating tumour cells”. These technologies provide the potential to monitor systemic tumour-cell dissemination in the blood and bone marrow as one of the first crucial steps in the metastatic cascade.

To date, it is not clear whether circulating tumour cell (CTC) measurements could replace examination of BM. BM aspiration is an invasive procedure and is hence more likely to cause complications, and it is frequently refused by the patients and avoided by the physicians

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Abbreviations: ALDH1 – aldehyde dehydrogenase receptor 1, BM – bone marrow, CKs – cytokeratins, CSCs – cancer stem cells, CTCs – circulating tumour cells, CXCR4 – chemokine receptor type 4, DTCs – disseminated tumour cells, EMT – epithelial-mesenchymal transition, EpCAM – epithelial cell adhesion molecule, EPISPOT – epithelial immunospot, ER – oestrogen receptor, FDA – US food and drug administration office, HER2 – human epidermal growth factor receptor 2, HIF-1 α – hypoxia-inducible factor 1 α , LN – lymph node, MAGE – melanoma-associated antigens, MBC – metastatic breast cancer, Muc1 – mucin 1, NT – neoadjuvant therapy, PB – peripheral blood, OS – overall survival, PCR – polymerase chain reaction, PR – progesterone receptor, VEGF – vascular endothelial growth factor, VEGFR1 – vascular endothelial growth factor receptor 1.

(Pierga et al., 2004). PB sampling is a relatively less invasive option with great potential for repeated evaluation used for the diagnosis of metastasis and monitoring of the treatment efficacy. Several different assays have been developed to detect disseminated tumour cells (DTCs) in the BM and CTCs in the PB.

The technical challenge for the detection of DTCs/CTCs is their extremely low occurrence in BM and BP (one tumour cell in 10^5 to 1×10^6 leukocytes) coupled with the task of correctly identifying the “event” as a tumour cell. Due to the rarity of the DTCs/CTCs, the existing techniques lack the sensitivity or efficiency to isolate these cells for further analysis. After successfully isolating the cells of interest, the second challenge is to reliably distinguish tumour cells from non-target haematopoietic cells in order to minimize the risk of false positives, which could generate poor clinical and therapeutic choices with a negative impact on the quality and/or expectancy of life in patients with cancer.

The most frequent methods – cytometric/immunological and molecular approaches will be briefly summarized. The methodology most commonly used to detect DTCs/CTCs is immunochemistry. This assay is based on immunocytochemical staining with monoclonal antibodies against epithelial or tumour-associated antigens (Gebauer et al., 2001; Gerber et al., 2001). To date, cytokeratins (CKs) have become the most widely accepted protein markers for the detection of epithelial tumour cells in mesenchymal tissues such as BM, blood or lymph nodes (Pantel et al., 1994; Braun et al., 2005). However, different staining techniques can result in specificity variations (Borgen et al., 1998). Immunocytochemical analysis is usually used in combination with density gradient centrifugation, immunomagnetic procedures or size filtration methods to enrich tumour cells prior to their detection (Paterlini-Brechot et al., 2000; Pinzani et al., 2006; Wong et al., 2006; Zach and Lutz, 2006). The advantage of this method is the possibility of further morphological analysis of the detected DTCs/CTCs.

The use of new automated devices for microscopic screening of the immunostained slides may help to read slides more rapidly and to increase reproducibility of the read-out (Borgen et al., 2001; Witzig et al., 2002; Kraeft et al., 2004). Among the commercially available systems, the CellSearch™ system (Veridex, Warren, NJ) has gained considerable attention because it allows both automated immunomagnetic epithelial cell adhesion molecule-based (EpCAM) enrichment and cytokeratin staining of CTCs in blood samples (Cristofanilli et al., 2004). This system was approved by the FDA for monitoring patients with metastatic breast, colon and prostate cancer.

A new technique, designated EPISPOT (epithelial immunospot), allows detection of viable DTCs/CTCs owing to their ability to secrete individual proteins after 48 h of short-term culture (Alix-Panabieres et al., 2007b). Most recently, a microfluid platform called the “CTC-chip”, which consists of an array of anti-EpCAM antibody-coated microspots capable of capturing CTCs

from unfractionated blood under controlled laminar flow conditions, was presented (Nagrath et al., 2007).

Molecular detection of DTCs/CTCs based on PCR amplification of either DNA or complementary DNA (mRNA) is hindered by the fact that the tumour cells of interest cannot be morphologically identified and isolated for further analyses. DNA-based methods rely on the detection of known mutations, amplifications or methylation patterns in the tumour cells. Although numerous genetic alterations have been described, because of the enormous heterogeneity of genetic alterations in tumour cells, currently no universally applicable DNA marker exists for their detection (Alix-Panabieres et al., 2007b; Pantel et al., 2008). Another possible confounder is the fact that DNA molecules are relatively stable, and therefore the detection of tumour-specific genomic aberrations may not necessarily indicate the presence of only intact DTCs/CTCs, but also DNA fragments derived from necrotic or apoptotic tumour cells.

1. Principle of development of distant metastasis, genesis of breast cancer stem cells, epithelial-mesenchymal transition and characterization of non-proliferating state called “dormancy”

1.1. Metastatic cascade

Cancer is a disease of cell proliferation, causing accumulation of cells, of differentiation, causing loss of structure and function, and of tissue organization, leading to invasion and survival in an ectopic environment. Carcinomas disseminate in the course of a multistep process referred to as metastatic cascade, of which the entry of cancer cells into the circulation (formation of the CTC pool) is a central event (Paterlini-Brechot et al., 2000; Mocellin et al., 2006). CTCs may reach multiple organ sites; however, their successful homing to organs and seeding as macro-metastasis is not random.

In this regard the “seed and soil” hypothesis first proposed by Paget over 100 years ago suggests that tumour cell retention and secondary growth is tissue/organ selective, a notion borne out in metastatic patterns of several human malignancies. Fidler (2003) proposed that metastatic cancer cells are selected for a high degree of efficiency in performing multiple biological tasks, properties that he described as a “decathlon champion” model. This includes successful invasion, intravasation (generation of CTC pool), survival in blood, embolization and arrest in a distant bed, and extravasation followed by ectopic growth. In addition to this selective process, metastasis also entails instructive and adaptive events, including interactions with tumour microenvironment stroma.

This broader view of the tumour cell dissemination process also encompasses their ability to initiate angiogenic, immune or inflammatory responses, all of which may impact the generation of CTCs and may vary be-

tween individual cancer patients based on their disease, age, comorbidities and genetic makeup (Fidler, 2003; Rak et al., 2008). There has been large data to indicate that metastases occur much earlier during tumour progression than once believed. Indeed, the progenitors of the later arising metastases must be present among those cells that have disseminated to distant sites before removal of the primary tumour.

In fact, based on the correlation of tumour size and time to metastasis, it is estimated that tumour cell dissemination may occur on average five years before diagnosis for breast cancer (Engel et al., 2003). It has been calculated that approximately 1×10^6 CTCs/g of tumour tissue are released daily into the circulation, but less than 0.1 % of CTCs will successfully settle in secondary organs (Dawood and Cristofanilli, 2007).

Klein et al. (2002) investigated the genetic heterogeneity of single DTCs (in blood, lymph node and bone-marrow tissues) by single-cell genomic hybridization in various cancer types. Unexpectedly, high genetic divergence was seen in minimal residual cancer disease, whereas in emerging clinically evident metastasis, heterogeneity was strikingly reduced. In another study, although genetically heterogeneous, DTCs from M0-stage patients displayed significantly fewer and different chromosomal aberrations than primary tumours or cells from M1-stage patients.

Thus, it was suggested that tumour cells may disseminate early in the cancer's history, and in a far less progressed genomic state than thought before. It is possible that they could acquire genomic aberrations typical of metastatic cells thereafter. In this way the idea that the precursors of metastasis are derived from the most advanced clone within the primary tumour was challenged (Schmidt-Kittler et al., 2003).

1.2. Breast cancer stem cells

Cancer stem cells (CSCs) are rare, self-renewing cells within the tumour mass, which also have a differentiation component and are required for initiation and maintenance of tumour growth (Clarke and Fuller, 2006; Trumpp and Wiestler, 2008). It is assumed that cancer stem cells can disseminate from the primary tumour to distant sites. This assumption is supported by the observation that primary tumour stem cells show an expression profile associated with metastatic relapse in patients with breast cancer (Liu et al., 2007).

CSCs are characterized by CD44 expression but low or undetectable levels of CD24 ($CD44^+CD24^{-/low}$). Moreover, expression of a new breast stem cell marker (ALDH1) was associated with poor clinical outcome in patients with breast cancer. Also, *in vivo* experiments showed that only ALDH1-positive cells were able to form metastasis in mice (Ginestier et al., 2007).

At least one study has confirmed a putative stem cell phenotype in DTCs (Ginestier et al., 2007), and another study has shown that the majority of early DTCs detected in the BM of breast cancer patients with a $CD44^+/CD24^-$ phenotype correlated with a higher prevalence of

bone metastasis (Abraham et al., 2005). As breast cancer stem cells have been shown to be generally triple-negative, triple-negative CTCs are in concordance with the cancer stem cell theory (Dontu et al., 2004).

1.3. Epithelial-mesenchymal transition

Epithelial cells provide cell-cell cohesion essential to maintaining the integrity of the multicellular organism and function as a critical barrier necessary for establishing a regulated internal environment, independent of the external environment (Shook and Keller, 2003). Cells exhibiting a mesenchymal phenotype provide support and structure to the epithelial cells particularly through production of an extracellular matrix and, unlike the rather confined and immobile epithelial cells, are highly motile and invasive (Hay, 2005).

Epithelial tumour cells running metastatic dissemination acquire a more motile and invasive phenotype and undergo epithelial-mesenchymal transition (EMT), which is promoted by transcription factor TWIST1 (Kang and Massague, 2004). TWIST1 has been part of the gene expression signature identified in EpCAM-enriched cells from BM of breast cancer patients after chemotherapy. TWIST1 expression, which was not observed in EpCAM-enriched cells of BM from healthy volunteers, correlated with the occurrence of distant metastasis and local progression, even in pretreatment BM samples (Watson et al., 2007).

EMT provides a mechanism for tumour cells to leave the primary tumour and invade the local tissue and blood vessels, setting the stage for metastatic spread. Therefore, EMT is hypothesized to contribute to tumour progression, and indeed clinical evidence suggests that regulators of EMT in cancer cells correlate with poor patient outcomes and tumour aggressiveness (Logullo et al., 2010).

1.4. Cancer dormancy

Disease-free periods can last from several years up to as long as 20–25 years in breast cancer patients (Karrison et al., 1999). This suggests that a pause in disease progression often occurs and might be explained by different forms of “dormancy”; this could be a common feature of cancer progression.

In 2007, Aguirre-Ghiso postulated two different states of „cancer dormancy“, tumour-cell dormancy and tumour mass dormancy (Aguirre-Ghiso, 2007). Tumour-cell dormancy occurs when single DTCs have entered a non-proliferative “quiescent” state, whereas tumour-mass dormancy describes a stage where cancer cells are more active and proliferate, but the growth of the tumour mass (that is, micrometastasis) is inhibited because an equal fraction of tumour cells undergo apoptosis. However, this conceptual framework is still under debate.

At present, little is known about the factors that might have a role in the “awakening” of dormant tumour cells that leads them into the dynamic phase of metastasis formation.

The steady state that regulates dormancy might be disturbed by both changes in DTCs (for example, addi-

tional mutations or epigenetic modifications in genes controlling cell proliferation and apoptosis) and the surrounding microenvironment (for example, release of growth and angiogenic factors, stress, immune system) (Aguirre-Ghiso, 2007).

The role of the immune system as a potentially important host component for controlling metastatic progression is still under debate. Koebel and co-authors highlighted the importance of immune surveillance for the process of tumour dormancy in an osteosarcoma mouse model. They showed that immunity can restrain cancer growth for extended time periods, called equilibrium. Escape and equilibrium are distinct. Whereas equilibrium represents the time of tumour cell persistence without expansion, escape is characterized by progressive tumour growth (Koebel et al., 2007).

Previously, Kaplan et al. (2005) demonstrated that bone-marrow-derived haematopoietic progenitor cells that express vascular endothelial growth factor receptor 1 (VEGFR1) are able to travel to tumour-specific pre-metastatic sites and form cellular clusters before the onset of tumour cells – which makes VEGFR an interesting target in the clinical setting. Angiogenesis is formation of a new blood supply from the pre-existing vasculature, and is stimulated by an angiogenic “switch” that occurs when the ratio of inducers to inhibitors tips in favour of inducers. There is ample preclinical evidence that the angiogenic switch is important for the escape from cancer dormancy and the subsequent formation of metastasis (Steeg, 2006). Using mouse models of pulmonary metastasis, Gao et al. (2008) identified bone-marrow-derived endothelial progenitor cells as critical regulators of this angiogenic switch.

Additional microenvironmental processes might influence the dormant state of DTCs and micrometastasis. For example, during inflammation and wound healing a plethora of cytokines are released, and some of these factors can induce migration and growth of epithelial tumour cells (Coussens and Werb, 2002). Among the protein characteristics, expression of tyrosine kinase receptor HER2 and the urokinase-type plasminogen activator receptor on DTCs/CTCs is correlated to metastatic relapse in breast cancer and gastric cancer, respectively (Heiss et al., 1995; Wulfing et al., 2006). Thus, signalling mediated by HER2 and urokinase-type plasminogen activator receptor might be important for the transition of DTCs from a dormant stage to an active growth phase, and future strategies aimed at inducing and/or maintaining tumour cell dormancy may include concomitant blocking of these proteins (Aguirre-Ghiso, 2007).

2. Disseminated tumour cells and circulating tumour cells – definition, molecular and functional characterization

Occult tumour cells of epithelial origin found in the BM and PB of breast cancer patients are termed DTCs

and CTCs, respectively. DTCs and CTCs are only rarely found in the BM and PB of otherwise healthy women. Several investigators have provided evidence that most detectable epithelial cells in the BM (Klein et al., 2002; Scharadt et al., 2005) or PB (Fehm et al., 2002) of women with breast cancer harbour genomic alterations characteristic of malignant cells.

DTCs/CTCs are rare cells. It is estimated that $\sim 1 \times 10^6$ tumour cells per gram of tumour tissue enter daily into the bloodstream (Paterlini-Brechot et al., 2000). This shedding is discontinuous, and detected CTCs are very heterogeneous, some destined to never succeed at implantation. Indeed, colonization of distant organs by CTCs is an extremely inefficient process, and the vast majority of these cells may either be destroyed in the circulation, or become dormant at distant sites due to the absence of proper growth regulatory niches. These cells often have low to absent expression of proliferation markers, but interestingly, they have been shown to retain capacity to divide in the presence of appropriate stimuli (Solakoglu et al., 2002). However, once metastasis is established, the subsequent seeding of cancer cells may become much more efficient and deadly (Jacob et al., 2007). Inherent to this is the amplification and change of the CTC pool during the sequential cycles of cancer cell dissemination.

While many questions surrounding these events remain unanswered, the accurate detection and characterization of CTCs may shed new light on the aggressiveness and metastatic potential of the underlying disease. All cytokeratin-positive cells should be classified as disseminated tumour cells, i.e. cytokeratin-positive cells with disseminated tumour cell morphology or cytokeratin-positive cells.

The morphological features of DTCs/CTCs are (Vincent-Salomon et al., 2008; Attard and de Bono, 2011):

1. The presence of cell clusters.
2. Large cell size with a clearly enlarged nuclear size and a high nuclear-to-cytoplasmic ratio, and strong or irregular cytoplasmic staining for cytokeratin.
3. Staining partially covers the nucleus. A large nucleolus can be seen and the nucleus is often granular or stippled.
4. EpCAM-positive.
5. Positive for CK8, 18, or 19.
6. Negative for CD45.
7. At least $4 \mu\text{m} \times 4 \mu\text{m}$ in size.

According to morphological classification guidelines, positivity rates are about 13–15 % (Naume et al., 2004) in contrast with the 30–35 % positivity rates reported in studies based exclusively on cytokeratin positivity without morphological analysis (Braun et al., 2005).

It has been shown that CTCs have a half-life of 1–2.4 h (Meng et al., 2004) and are non-replicating (Muller et al., 2005) and that they must be replenished by replicating cells from elsewhere. Potentially, this could be the BM; however, the DTCs, when in the BM,

are also non-replicating in the majority of primary breast cancer patients.

The vast majority of DTCs in BM and CTCs in blood appear to persist in a non-proliferating state, which was shown by Ki-67 negativity (Pantel et al., 1993; Muller et al., 2005). Furthermore, only half of the breast cancer patients with DTCs relapse, whereas the other half remains tumour-free over a 10-year follow-up period (Braun et al., 2005). On the other side, this dormant state of DTCs/CTCs might also be the cause for the lack of effect of adjuvant chemotherapy on the elimination of these cells in high-risk breast cancer patients (Braun et al., 2005).

In order to escape from the dormant state into the dynamic phase of metastasis formation, dormancy has to be disturbed probably by both genetic and epigenetic changes in the DTCs/CTCs as well as in the surrounding microenvironment or pre-metastatic niche (Vessella et al., 2007). However, conditions and timing of outgrowth of dormant tumour cells are not known thus far (Paterlini-Brechot et al., 2000; Alix-Panabieres et al., 2007a).

Although there is evidence for a molecular signature of primary tumours spreading early into BM (Woelfle et al., 2003), there is only limited information about global gene expression analyses of DTCs/CTCs. In DTCs, heterogeneity with respect to the expression of growth factor receptors, adhesion molecules, proteases and their inducers and receptors, major histocompatibility complex antigens, signalling kinases, melanoma-associated antigens (MAGE), or telomerase activity has been observed (Pantel et al., 1991; Klein et al., 2002; Pantel and Brakenhoff, 2004). Most DTCs and CTCs were detected in a non-proliferative state, as revealed by Ki-67 immunostaining (Muller et al., 2005). Expression of hypoxia-inducible factor 1 α (HIF-1 α), vascular endothelial growth factor (VEGF), and VEGF-receptor (VEGFR2), associated with angiogenesis and tumour progression, was shown in CTCs and DTCs of breast cancer patients (Kallergi et al., 2009). The first hints for stem cell features of DTCs in BM were provided by Balic et al. (2006) and by Alix-Panabieres et al. (2007a), who demonstrated a significant number of DTCs from BM of breast cancer patients with either CD44⁺/CD24^{-low} or CK19⁺/Muc-1⁻ stem cell-like phenotypes.

The human epidermal growth factor receptor 2 (HER2), the expression of which in primary tumours is the basis for trastuzumab treatment decisions of breast cancer patients, has gained particular interest. HER2 is a transmembrane tyrosine kinase receptor encoded by a proto-oncogene located on chromosome 17q21. The HER2 proto-oncogene is amplified or over-expressed in approximately 20 % of invasive primary breast cancers (Coussens et al., 1985; Slamon et al., 1989). A positive HER2 status has been linked with aggressive tumour behaviour and resistance to cytotoxic and endocrine therapy (Konecny et al., 2003; Moliterni et al., 2003). HER2 is responsible for an increase in proliferation and sur-

vival of the primary tumour and also plays a role in the distant but not completely transformed lesions. An increase in motility of intravasating and extravasating cells, a decrease in apoptosis, enhancement of communication with the microenvironment, and regulation of adhesion alters these distantly migrated cells. HER2 over-expression enhances the transformation potential. Current understanding of how untransformed, stem/progenitor cell-like, HER2-expressing cells metastasize and how they could be therapeutically targeted to prevent HER2⁺ breast cancer progression should lead to new treatment schedules of targeted antibodies that are directed at incompletely transformed cells (Freudenberg et al., 2009). It was established that patients with HER2 amplification and/or over-expression are eligible for HER2-targeted treatment. As a consequence, strategies for response prediction and monitoring are of high clinical relevance.

In recent years, there has been a growing body of evidence that the HER2 status of the primary tumour may be different from metastatic disease and changes might occur during the treatment. A discrepancy between the primary tumour and distant metastasis has been observed in 7–26 % of cases (Gancberg et al., 2002; Regitnig et al., 2004; Vincent-Salomon et al., 2007; Simmons et al., 2009). In many patients with metastatic breast cancer, re-evaluation of the HER2 status by tissue biopsy of the metastatic lesion is not feasible due to the location of the metastatic site. In addition, the HER2 status may vary between different metastatic sites and also change during the treatment. Therefore, determination of the HER2 status of CTCs might be a strategy with potential clinical application.

These observations might have clinical relevance when selecting patients for HER2 targeted therapy. Patients with HER2-negative tumours but HER2-positive CTCs might also benefit from HER2-targeted therapy. In a metastatic setting, Meng and colleagues have already shown that metastatic patients who were regarded as HER2-negative on the basis of HER2 expression of their primary tumour had circulating HER2-positive cells and responded to trastuzumab (Meng et al., 2004). Similarly, the hormonal status of DTCs and CTCs could be completely different from that of the primary tumour, which on the one hand (tumour-negative, DTC/CTC-positive) could increase the number of patients eligible for endocrine therapy and on the other (tumour-positive, DTC/CTC-negative) could explain why endocrine therapy fails in a subset of hormone receptor-positive patients.

Ditsch and colleagues, in an observational study looking at 17 primary tumours and their corresponding DTCs, found that only two out of 11 patients (18 %) with oestrogen receptor α (ER α)-positive primary tumours had ER α -positive DTCs (Ditsch et al., 2003). It was demonstrated in a cohort of 254 patients with primary breast cancer that the primary tumour and DTCs in BM displayed a concordant ER α status in only 28 % of cases (Fehm et al., 2008). In summary, all published

the presence of brain metastasis (OR: 6.17, 95% CI = 2.14–17.79; $P = 0.001$), and inversely correlated with bone metastasis (OR: 0.47; 95% CI = 0.27–0.80; $P = 0.01$). In multivariate analysis, hormone receptors, number of metastatic sites and lines of therapy were independent prognostic factors for OS in patients without detectable CTCs. Patients without detectable CTCs before starting a new line of therapy comprise a heterogeneous group with substantially different prognosis. Authors showed that some important metastatic disease characteristics are predictive of undetectable CTC status in MBC.

Repeated BM sampling is considered the standard of care in patients with leukaemias or lymphomas, but it seems to be difficult to introduce in the clinical management of patients with solid tumours. Sequential peripheral blood analyses are more convenient to assess. Therefore, an important question is whether BP sampling can replace BM aspiration for the evaluation of minimal residual disease.

Wiedswang et al. (2006) compared the prognostic value of CTCs versus that of DTCs detected by immunocytochemistry in 341 breast cancer patients with sampling performed at a median follow-up of 40 months after the initial operation. Although both CTCs (10 % of the patients) and DTCs (14 % of the patients) were significantly associated with clinical outcome, DTCs were more informative than CTCs. By contrast, Bidard et al. (2008) reported superior significance of CTC counts, but they only analysed data from 37 patients with metastatic (stage M1) breast cancer. The results of these findings do not support the use of DTCs in BM as a replacement for detection of CTCs from blood as a prognostic indicator in breast cancer. Future studies on larger cohorts of patients, however, might help to clarify this important issue.

The clinical use of CTC measurements in patients with metastatic breast cancer is now being prospectively addressed in a randomized trial, SWOG-S0500, led by the Southwest Oncology Group (National Cancer Institute, 2011). This trial is expecting to enrol 500 patients with metastatic breast cancer, and it aims to determine whether patients with elevated CTC levels after three weeks of first-line chemotherapy show an improved OS and progression-free survival when changing to an alternative chemotherapy regimen at the next course, rather than waiting for clinical evidence of progressive disease. All patients will undergo blood collection before their first course of first-line chemotherapy to determine baseline CTC counts. Patients with a high risk of early progression (≥ 5 CTCs per 7.5 ml blood after completing one course of chemotherapy) will be stratified according to HER2 status and disease-type (bone-only versus measurable disease), and will be randomly allocated to one of two treatment arms: to continue their current chemotherapy regimen or switch to a different chemotherapy regimen. All patients who receive hormonal therapy or biological therapy and chemotherapy will continue to receive these therapies regardless of CTC levels.

The German Breast Group has conducted a successful clinical trial in the neoadjuvant setting. The GeparQuattro study (Riethdorf et al., 2010) is a phase III trial programme that incorporated different neoadjuvant therapy approaches with the addition of trastuzumab into current neoadjuvant regimes for primary breast cancer. This study was aimed at detecting and characterizing CTCs before and after neoadjuvant therapy (NT) in the peripheral blood of patients with breast cancer. The authors used the Food and Drug Administration-approved CellSearch™ system for CTC detection and evaluation of HER2 expression and developed HER2 immunoscore for CTCs. They detected ≥ 1 CTC/7.5 ml in 46 of 213 patients (21.6 %) before NT and in 22 of 207 patients (10.6 %) after NT ($P = 0.002$). Twenty (15.0 %) initially CTC-positive cases were CTC-negative after NT, whereas 11 (8.3 %) cases were CTC-positive after NT, although no CTC could be found before NT. CTC detection did not correlate with primary tumour characteristics. Furthermore, there was no association between tumour response to NT and CTC detection. HER2-overexpressing CTCs were observed in 14 of 58 CTC-positive patients (24.1 %), including eight patients with HER2-negative primary tumours and three patients after trastuzumab treatment. CTCs scored HER2-negative or weakly HER2-positive before or after NT were present in 11 of 21 patients with HER2-positive primary tumours. HER2 over-expression on CTCs was restricted to ductal carcinomas and associated with high tumour stage ($P = 0.002$) (Riethdorf et al., 2010)

Conclusions

The fact that breast cancer is not a uniform cancer entity but consists of several different subtypes with different molecular profiles, biological behaviour, and risk profiles poses a challenge for the clinical management. Prognostic and predictive factors constitute important tools for the individualization of breast cancer therapy to provide efficient treatment and to spare patients with excellent low-risk profiles from unwanted side effects of overtreatment. The lack of standardization for DTC/CTC detection and high intra- and interlaboratory differences in the results have additionally complicated the introduction of DTC/CTC analysis into the clinical practice. Standardization and automation are also pivotal to ensure high-throughput analyses as a precondition for clinical application.

Information about the DTC/CTC status may be used to assess prognosis of cancer patients and to stratify the patients at risk to systemic therapies aimed to prevent recurrences and metastatic relapses. Furthermore, DTC/CTC measurements within clinical trials might serve as an important biomarker for real-time monitoring of the efficacy of systemic therapies in individual cancer patients, and might thereby support accelerating drug development and defining subpopulations of patients with the highest treatment benefit.

data suggest that CTCs and DTCs may represent a unique and heterogeneous cell population.

3. Overview of several interesting studies investigating DTCs/CTCs and examples of ongoing clinical trials

Several studies over the last two decades have assessed the prevalence and prognostic value of micrometastatic dissemination of breast cancer cells. At present, the largest database exists for patients with breast cancer. A pooled analysis of data from European and US groups has confirmed the prognostic impact of DTCs in the BM of patients with breast cancer (1a level of evidence). The analysis of 4,703 patients, using conventional staging procedures such as bone scans, showed that approximately 30 % of women with primary breast cancer harboured DTCs in their BM at primary diagnosis in the absence of any signs of overt metastasis. The extended 10-year follow-up of these patients revealed an unfavourable prognosis compared with patients without DTCs (Braun et al., 2005).

In total, 1,499 patients received only hormonal therapy, 1,596 received cytotoxic therapy, 119 received combined hormonal-cytotoxic therapy, and 1,489, of whom 1,036 patients had stage pT1N0 disease, had no therapy. The presence of micrometastasis was a significant prognostic factor with respect to poor overall survival and breast-cancer-specific survival (univariate mortality ratios, 2.15 and 2.44, respectively; $P < 0.001$ for both outcomes) and poor disease-free survival and distant-disease-free survival (incidence-rate ratios, 2.13 and 2.33, respectively; $P < 0.001$ for both outcomes). In the multivariate analysis, DTC detection was an independent predictor of poor outcome. In the univariate subgroup analysis, breast-cancer-specific survival among patients with micrometastasis was significantly shortened ($P < 0.001$ for all comparisons) among those who received adjuvant endocrine treatment (mortality ratio, 3.22) or cytotoxic therapy (mortality ratio, 2.32). Moreover, breast-cancer-specific survival was significantly shortened among patients who had tumours no larger than 2 cm in diameter without lymph-node metastasis, and who did not receive systemic adjuvant therapy (mortality ratio, 3.65).

The study of Balic et al. (2006) represents an important first step in showing that micrometastases isolated from the BM of early-stage breast cancer patients are highly enriched for cells that display the cell surface markers $CD44^+/CD24^{-low}$, showing that the majority of early DTCs in BM have a putative breast cancer stem cell phenotype. It also suggests that these cells may display biological properties facilitating their metastatic spread, enabling them to colonize distant sites: (a) increased angiogenic capacity and (b) expression of receptors, such as CXCR4 (Wicha, 2006). In addition, a subpopulation of viable DTCs that are $CK19^+MUC1^-$ has also been previously suggested as breast stem cell-

like cells (Alix-Panabieres et al., 2007a). Thus far, it is, however, still unclear whether DTCs have self-renewal ability, the hallmark of stem cells.

In another study arranged by Krishnamurthy et al. (2010) authors investigated the occurrence of CTCs and DTCs in women with early stage breast cancer and evaluated the correlation of their presence with other prognostic markers. Blood and BM aspirations of 92 patients were collected at the time of primary breast surgery. CTCs were detected by using the CellSearch™ assay, and DTCs were detected by immunostaining BM aspirates for pancytokeratin. The presence of CTCs and DTCs was correlated with tumour classification (T1 vs T2), tumour histologic grade, ER status, progesterone receptor (PR) status, HER2 status, and lymph node (LN) status. These authors observed that CTCs and DTCs in women with early stage breast cancer did not correlate with the standard prognostic indicators that were considered.

Pachmann et al. (2005) evaluated the role of CTC levels in patients with early stage breast cancer receiving primary systemic chemotherapy. The levels of CTCs were determined before each session of the first three courses of chemotherapy. The authors noted that response of the tumour to chemotherapy was associated with a reduction of CTC levels. Furthermore, the reduction in the number of CTCs with chemotherapy strongly predicted for the final tumour size, determined at the time of surgery. These results suggest that CTCs could potentially be used to monitor response early on, preventing unnecessary toxicity by discontinuing non-effective treatment. Prospective trials would need to confirm the use of CTCs in this setting.

A number of studies have also looked at the HER2 in primary breast tumours and associated CTCs. These studies reported that up to one third of patients whose primary tumours do not overexpress HER2 have CTCs with amplified HER2. In view of the fact that trastuzumab, a humanized antibody against HER2 receptor, is known to have a substantial positive impact on the survival of patients with HER2-positive disease in both the adjuvant and metastatic setting, monitoring of CTCs for HER2 could help to identify patients that would not otherwise receive trastuzumab due to a negative HER2 status of the primary tumour to benefit from this therapy (Hayes et al., 2002; Fehm et al., 2010).

CTCs are an independent prognostic factor in metastatic breast cancer patients (MBC). However, CTCs are undetectable in one third of the patients. The aim of the study conducted by Mego et al. (2011) was to assess the prognostic factors in MBC patients without detectable CTCs. This retrospective study included 292 MBC patients. CTCs were enumerated before patients started a new line of treatment using the CellSearch™. Overall survival (OS) was calculated from the date of CTC measurement and estimated by the Kaplan-Meier product limit method. CTCs were not detected in 35.96 % patients, while 40.75 % patients had CTCs ≥ 5 . Undetectable CTC status was positively correlated with

Given the development of new targeted molecular therapies, there will be continuing need for identifying and devising new markers that will be able to predict a specific response. It will be a challenge for scientists and clinicians to select the most promising ones particularly where over-expression of the target is not required for activity. With the effort being exploited in this area and the enormous strides being made in characterizing the molecular characteristics of individual cancers, the future should provide us with unique case-specific patterns of biomarkers, which will help to optimize tailored therapies and individualize breast cancer patient care. Special attention needs to be paid to the design and conduct of clinical trials, which have the potential to validate emerging biomarkers for their clinical application.

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