

Disseminated Tumor Cells in Bone Marrow and Circulating Tumor Cells in Blood of Breast Cancer Patients: Current State of Detection and Characterization

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Key Words

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Abstract

Despite the progress resulting from early detection and improved adjuvant therapy, the prognosis of breast cancer patients is still limited by the occurrence of distant metastases largely due to clinically occult micrometastases that remain undetected at primary diagnosis even by high-resolution imaging approaches. Recent research efforts have concentrated on the identification of additional parameters allowing individual risk assessment and stratification of patients for targeted therapies, since traditional prognostic factors are not sufficient to predict metastatic relapse and treatment decisions are still mainly based on statistical risk parameters. Highly sensitive and specific immunocytochemical and molecular assays now enable the detection and characterization of disseminated and circulating tumor cells (DTCs and CTCs, respectively) at the single cell level in bone marrow (BM) and peripheral blood, providing insights into the first crucial steps of the metastatic cascade. However, because of the still high variability of results in DTC/CTC detection, the necessity of standardized approaches will be discussed. A large number of studies showed that the presence of DTCs in BM has prognostic impact for primary breast cancer patients. DTCs are likely to escape from chemotherapy by

maintaining a dormant nonproliferating state. There is also evidence for a stem cell-like phenotype of DTCs, probably contributing to the opportunity to escape from dormancy control and to start expansion to manifest metastases. Blood would also be an ideal source for the detection and monitoring of CTCs because of an easy noninvasive sampling procedure enabling repeated analyses. While prognostic significance of CTCs could be reliably demonstrated for metastatic breast cancer, studies to analyze the impact of CTCs in primary breast cancer patients and the potential to replace or supplement BM analysis are still ongoing. Furthermore, molecular characterization of CTCs might contribute to improving targeted and more individualized cancer therapies.

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Introduction

The occurrence of distant metastases is the main cause of death for breast cancer patients. However, crucial factors forcing cancer cells to move and grow outside of the primary organ are still not well understood [1]. An association of breast cancer and bone metastasis was already described in 1889 with the publication of Steven Paget's theory of seed and soil [2]. Breast cancer-related metastasis in bone marrow (BM) was also suggested by Rohr and Hegglin [3], who identified metastatic cells in BM biopsies by hematoxylin and eosin staining. When Schreiber

et al. [4] described the first single disseminated tumor cells in BM smears in nonmetastatic breast cancer patients, only a few publications dealt with micrometastasis [5]. Moreover, morphological criteria were not sufficient to undoubtedly distinguish single epithelial tumor cells from BM cells, especially because of the wide variety of morphologically uneven hematopoietic and mesenchymal stem as well as progenitor cells [6].

Important progress in the field of BM micrometastasis arose from the introduction of immunocytochemical staining procedures using antibodies against epithelial-specific markers (EMA, cytokeratins) that were not expressed on the surrounding BM cells [7]. Using these methods, it became more and more accepted during the last 2 decades that BM is a common homing and surviving organ not only for breast cancer cells but also for cancer cells from other organs [8, 9]. These cells are likely to escape from the host immune system in a dormant state until internal and/or external signals might enable them to move and grow out to overt metastases at different organs [10–14].

In this review, we focus on modern sensitive and specific detection methods for disseminated and circulating tumor cells (DTCs and CTCs, respectively) as well as their clinical relevance. We will further concentrate on the question whether and when BM examination can be replaced and supplemented by blood analysis. Furthermore, we will present new data about the phenotypical and molecular characterization of DTCs and CTCs under particular consideration of cancer stem cell features and potential therapeutically relevant target molecules.

Detection of DTCs in Bone Marrow

Current models of breast cancer metastasis support the possibility of an early detachment of cells from primary tumors and the direct release of DTCs into blood and BM, bypassing in some cases the lymphatic system [1, 13]. DTCs are rare with only 10–20 cells among millions of BM cells. In order to increase the chance to detect DTCs in this organ, procedures had to be developed for their enrichment prior to detection and further characterization. To this end, different density gradient centrifugation methods such as Ficoll-based assays or the OncoQuick approach, as well as positive or negative immunomagnetic enrichment procedures and simple filtration methods separating tumor cells by their size, have been established [13, 15].

Today, in general, there are 2 different methods to screen BM aspirates for DTCs, namely cytologic/cytometric (antibody-based) and molecular approaches [13, 15, 16]. Among the cytologic methods that allow isolation and enumeration of individual cells, immunocytochemistry is the most widely used approach [1, 15]. Because of the absence of tumor-specific target antigens, most frequently antibodies against various epithelium-specific antigens such as cytoskeleton-associated cytokeratins, surface adhesion molecules or growth factor receptors are applied for the detection of carcinoma cells [15–17]. The main advantage of cytologic methods is the opportunity to combine immunostaining with the morphology of the cells so that both cell size and shape as well as the nucleus-plasma relation might be estimated and illegitimate expression of the protein of interest in BM cells can be excluded as far as possible. The detection of DTCs in BM is still not part of the routine tumor staging in the clinical practice, but emerging data anticipate a future role of DTC detection for risk stratification and therapeutic monitoring of breast cancer patients [18–20]. However, the detection rates of DTCs in BM from nonmetastatic breast cancer patients vary considerably [21, 22]. This might reflect the different sensitivity, but also specificity, of the numerous detection methods and marker genes/proteins used thus far. The recently defined consensus concept for the detection of DTCs in BM, signifying enrichment of mononuclear cells from BM by Ficoll density gradient centrifugation and immunocytochemical detection of cytokeratin expression as standard procedure, should help overcome these problems and provide the basis for future multicentric clinical trials [23]. The authors recommend the pan-anti-cytokeratin antibodies A45-B/B3 or AE1/AE3 against a wide spectrum of cytokeratins as standard application, thereby ensuring detection of DTCs also in cells that have downregulated the expression of individual cytokeratins in the course of epithelial-mesenchymal transition [23]. Microscopic screening of large amounts of immunostained cytological preparations is accomplished by automatic microscopes using sophisticated imaging approaches. Criteria to evaluate morphology and staining results have also been defined to avoid false-positive and false-negative results [23–26]. Despite existing recommendations for standard operation procedures, however, there are still limitations to the standardization of immunocytochemical methods with respect to reproducibility of the staining procedure itself as well as microscopic interpretations. Therefore, both intra- and interlaboratory evaluation of the methods is required to ensure reliability of the results [26].

Besides immunocytochemical methods, very sensitive nucleic acid-based techniques now enable the detection of DTCs also at the single cell level. The main advantage of these methods is the nearly unlimited availability of primers for almost every gene of interest. Although numerous genetic alterations have been described in breast cancer cells, heterogeneity is enormous, so that at present no universally applicable DNA marker exists for the primary screening of a wide range of DTCs [13, 15].

Further efforts have been made to detect free circulating DNA or epigenetic alterations of circulating DNA such as methylation in BM and blood plasma, but the results are still preliminary [27, 28]. Furthermore, it is unclear whether the amplified DNA derives from DTCs/CTCs or is being shed from tumor or normal tissue [29].

Therefore, measurement of epithelium-specific or more organ-specific mRNA species such as cytokeratin 19 or mammaglobin mRNA by RT-PCR has been proven as promising approach to detect DTCs in BM samples [30–35]. Because of the absence of tumor-specific markers, the main drawback of using surrogate tissue-specific markers, however, are false-positive results due to illegitimate low-level transcription of epithelial or breast tissue-specific genes in normal cells [29, 36]. Furthermore, heterogeneity in the expression of particular genes is not recognizable and the expression level of a gene of interest per cell cannot be estimated. Current analyses are mainly performed by quantitative real-time RT-PCR, ensuring the discrimination between different levels of expression. Moreover, multimarker real-time RT-PCRs have the potential to improve the method even in case of downregulation of the expression of a single gene [22, 37–39]. However, storage and sample preparation have to be performed under conditions avoiding RNA degradation, one of the major problems of RT-PCR approaches [35]. The application of multimarker assays might also compensate for low mRNA amounts due to the low number of tumor cells. There are several excellent reviews listing the marker genes currently used in RT-PCR approaches to detect DTCs in BM or CTCs in blood from breast cancer patients [15, 29, 40].

The methods described above are not able to discriminate between viable and apoptotic DTCs. A new technique, designated EPISPOT (epithelial immunospot) offers the advantage of detecting viable tumor cells by their ability to secrete individual proteins. In a recently published study it could be demonstrated that BM samples from metastatic and nonmetastatic breast cancer patients

contain viable tumor cells which secrete Muc-1 and/or cytokeratin 19 in about 90 and 50% of cases, respectively, whereas in controls from healthy women, cells secreting these proteins could not be detected [41].

Clinical Relevance of DTCs in Bone Marrow

A large number of studies have documented DTCs in BM from patients with most types of epithelial cancers [1, 8]. Within the last 15 years, several studies have demonstrated that detection of DTCs in BM of breast cancer patients is accompanied by a substantially worse prognosis [42–44]. In a pooled analysis evaluating the results from 9 different European centers, including a total of 4,703 patients, Braun et al. [19] have reported that approximately 30% of women with primary breast cancer have DTCs in BM, and in a multivariate analysis, the 10-year follow-up of these patients revealed a significantly decreased overall survival, when compared to patients without DTCs. The presence of DTCs in BM was significantly associated with higher tumor stage, worse differentiation, lymph node metastasis and negativity in hormone receptor expression. Prognostic relevance was shown for all subgroups, even among those patients with small tumors and without lymph node metastasis. Although using different antibodies and detection methods, almost all investigators participating in this pooled analysis used anti-cytokeratin antibodies to screen for DTCs in the BM [19].

Moreover, Wiedswang et al. [45] and Janni et al. [20] demonstrated that the persistence of DTCs in BM from breast cancer patients after adjuvant therapy is predictive for a subsequent disease recurrence. However, only prospective clinical trials will show whether patients with persistent DTCs will benefit from a second-line adjuvant treatment, for example by bisphosphonates or antiangiogenic therapeutics.

Can Bone Marrow Examination for DTCs Be Replaced by Blood Analysis for CTCs?

Aspiration of BM is invasive, time-consuming and in many cases painful or at least uncomfortable for the patients, precluding repeated samplings necessary for therapy-monitoring studies. Moreover, BM aspiration is more difficult to standardize with regard to the required volume and quality. Consequently, recent efforts have concentrated on the detection of CTCs in peripheral blood

(PB) of cancer patients [29], but the clinical usage of CTCs has not yet been implemented for routine clinical practice. Furthermore, there are only a limited number of studies comparing BM and PB examinations performed at the same time points, and the clinical significance of CTCs in PB is less clear than that for DTCs in BM. In all studies published thus far, there was a higher frequency of BM-positive than blood-positive samples from the same patients [46–49], probably due to the fact that BM might provide conditions for homing and survival of DTCs, thus contributing to their accumulation in this compartment.

Although both Pierga et al. [46] as well as Muller et al. [47] reported about a significant number of patients with concordant results concerning BM and blood analysis, in the study of Pierga et al. [46] only the presence of DTCs in BM and not that of CTCs in blood had prognostic relevance for disease-free survival in nonmetastatic breast cancer patients. In a study reported by Wiedswang et al. [48] on 341 breast cancer patients with median 40 months of follow-up after surgery, both CTCs and DTCs were significantly associated with disease-free survival; however, considering only node-negative patients, DTCs but not CTCs predicted differences in disease-free survival. Interestingly, the presence of both DTCs in BM and CTCs in blood in a subgroup of patients resulted in an especially poor prognosis [48]. While all studies mentioned above applied immunocytochemical methods, also real-time RT-PCR detection of DTCs in BM had superior prognostic significance in comparison with CTCs in patients with breast cancer [49]. In the study reported by Benoy et al. [49], cytokeratin 19 and mammaglobin mRNA levels were analyzed by quantitative RT-PCR.

Currently, the results obtained by comparative studies do not support a replacement of BM by blood analysis, but CTC detection might have supplementary value. There is an increasing number of studies demonstrating clinical relevance of CTCs in blood detected by real-time RT-PCR identifying either only cytokeratin 19 mRNA or multiple markers [15]. Recently, analyzing cytokeratin 19 mRNA by real-time RT-PCR, Xenidis et al. [50] reported about CTCs detected in 22% of blood samples from 167 node-negative breast cancer patients as significantly associated with overall and disease-free survival. A correlation of the presence of CTCs in blood to the lymph node status was found in 2007 by Nakagawa et al. [51], who detected CTCs with the help of a multimarker real-time RT-PCR in 39/90 (43%) stage I–III breast cancer patients, but not in normal healthy volunteers.

The lack of standardization for CTC detection and high intra- and interlaboratory differences in the results have additionally complicated the introduction of PB testing into the clinical practice. Standardization and automation are also pivotal to ensure high-throughput analyses as a precondition for clinical application and multicenter studies. Important progress in this field arose from the development of an automated enrichment and immunocytochemical detection system for CTCs (CellSearch™) [52, 53]. This system consists of an automated instrument for the enrichment of epithelial cells by ferrofluids coated with anti-EpCAM antibodies followed by immunostaining of captured cells with fluorescently labeled anti-cytokeratin and anti-CD45 antibodies (AutoPrep), and a semi-automated microscope for scanning and reading results (CellSpotter® Analyzer). Using this system, Cristofanilli et al. [52, 53] demonstrated in a prospective study that CTC detection provided significant prognostic information for patients with metastatic breast cancer. Additionally, Hayes et al. [54] demonstrated that CTCs at each follow-up time point during therapy of these metastatic breast cancer patients predict progression-free and overall survival. The CellSearch system has been cleared by the US Food and Drug Administration for routine clinical use in metastatic breast cancer patients. Validation data from 3 independent laboratories and high interinstrument accordance confirmed the reliability of this system for CTC measurements in PB from metastatic breast cancer patients. Furthermore, it was shown that samples can be shipped at room temperature and CTC counts are stable for at least 72 h, which facilitates testing at central laboratories or remote sites requiring transportation [55].

There are also several reports about the detection of CTCs in patients with primary breast cancer, however, mostly with lower frequencies and varying results concerning both the number of positive patients as well as the number of CTCs in individual patients [47, 50, 51, 56–58].

Phenotypical and Molecular Characterization of DTCs in Bone Marrow and CTCs in Blood

The characterization of DTCs/CTCs is aimed to (1) provide evidence for their malignant origin and (2) identify further diagnostically and therapeutically relevant features of these cells, which might enable a more targeted and individualized antimetastatic therapy. This characterization is hampered by the fact that DTCs/CTCs can exhibit features distinct from the primary tumors, but on

the other side this could help to identify cancer patients for additional targeted therapies.

By multiple fluorescence in situ hybridization analysis, Fehm et al. [59] could show that the vast majority of CTCs in blood from breast cancer patients are aneuploid and derived from the primary tumor. By single cell comparative genomic hybridization, Klein et al. [60] indicated that DTCs might be genomically unstable and heterogeneous. Moreover, Schmidt-Kittler et al. [61] suggested that DTCs from BM of breast cancer patients disseminate in a less progressed genomic state and might acquire genomic alterations typical for metastatic cells later.

The vast majority of DTCs in BM and CTCs in blood appear to persist in a nonproliferating state which was shown by Ki-67 negativity [10, 47]. Furthermore, only half of the breast cancer patients with DTCs relapse, whereas the other half remains tumor free over a 10-year follow-up period [19]. 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 [18]. 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 premetastatic niche [14, 62, 63]. However, conditions and timing of outgrowth of dormant tumor cells are not known thus far [1, 13]. Although there is evidence for a molecular signature of primary tumors spreading early into BM [64], there is only limited information about global gene expression analyses of DTCs/CTCs. Transcriptional analyses of EpCAM-enriched BM and blood cells resulted in gene expression profiles that may be used to distinguish normal donors from cancer patients [65, 66]. Further studies have to elucidate whether individual genes, the expression of which is changed in these cell populations, might become markers to recognize recurrence in breast cancer patients early [66]. Interestingly, TWIST1, a transcription factor that previously has been identified to play an important role in metastasis by promoting epithelial-mesenchymal transition [67–70], was part of the gene expression signature identified in EpCAM-enriched cells from BM of breast cancer patients after chemotherapy [66]. 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 [66].

DTCs/CTCs seem to be heterogeneous with regard to the expression of growth factor receptors, adhesion molecules, proteases and their inducers and receptors, major histocompatibility complex antigens or signaling kinases [11, 71–76]. Of particular interest is the epidermal growth factor receptor HER2, the expression of which in primary tumors forms the basis of Herceptin treatment decisions for breast cancer patients. As shown by Braun et al. [77], HER2 overexpression on DTCs in BM was predictive for a poor clinical outcome of stage I–III breast cancer patients. While Vincent-Salomon et al. [78] showed on 27 breast cancer patients that the HER2 status remained relatively stable between primary tumors and BM micrometastases in most cases, there is also increasing evidence for discrepancies between the HER2 status in primary tumors and DTCs in BM [79]. Solomayer et al. [79] detected HER2-positive DTCs in 12/20 BM samples from patients with HER2-negative primary tumors. Although HER2 expression was heterogeneous in DTCs from individual patients, HER2-positive DTCs might identify additional patients who can benefit from Herceptin therapy. The HER2 status of CTCs from PB might also be different from that of the corresponding primary tumors as reported by Wulfing et al. [80]. These authors presented a significant number of patients whose primary tumors were HER2 negative, whereas CTCs were HER2 positive before surgery [80]. Moreover, in this study the detection of HER2-positive CTCs correlated significantly with disease-free and overall survival [80]. It remains to be investigated whether high levels of HER2-positive CTCs reflect the activity of the tumor and have predictive value for an improved response of the patients to Herceptin treatment [80]. Although Meng et al. [81] reported a high agreement (97%) of the HER2 status between primary tumors and CTCs in 31 cases, during tumor progression HER2-positive CTCs could be detected in 9 of 24 breast cancer patients in spite of HER2-negative primary tumors. These CTCs might have acquired HER2 gene amplifications. Four of these patients received Herceptin therapy and 3 of them responded to this therapy [81]. In the study reported by Apostolaki et al. [82], adjuvant chemotherapy eliminated HER2 mRNA-positive CTCs in 16/45 patients. The detection of HER2 mRNA-positive CTCs after chemotherapy was associated with a reduced disease-free survival. Moreover, in 8/161 patients with HER2-negative primary tumors, HER2 mRNA-positive CTCs could be detected [82]. Thus, the detection of HER2 mRNA-positive CTCs after adjuvant chemotherapy in the PB of stage I and II breast cancer patients might provide information

about the efficacy of chemotherapy and the prognosis of the patients and identify patients in need of additional Herceptin therapy [82].

Detection of Stem Cell-Like Phenotypes

The hypothesis that breast cancer might be originated from tissue stem or progenitor cells exhibiting the capability to self-renew and differentiate has gained increased attention during the last years [83–86]. Among the various features described for breast cancer stem cells, CD44 positivity and absence or weak expression of CD24 seem to be characteristic for breast cancer founder cells with a higher capacity to form tumors in immunosuppressed mice than other subtypes of breast cancer cells [87–89]. There is also evidence for CD133-positive stem cells in breast cancer [90, 91]. Gene expression analysis of CD44⁺/CD24^{-/low} cells separated from CD44-negative cells of breast cancer tissue resulted in a 186-gene invasiveness gene signature which was significantly associated with overall and metastasis-free survival of breast cancer patients [92]. Furthermore, CD44⁺/CD24^{-/low} breast cancer cells have upregulated different genes including Notch [93]. Very recently, Farnie et al. [94] demonstrated that aberrant activation of Notch signaling is an early event of breast cancer development. Apart from the Notch signaling pathway, other developmental pathways such as Wnt and hedgehog have been described to play a role in regulating cancer stem cell features [95, 96].

There are first hints that DTCs in BM also express stem cell features, as the majority of them are CD44⁺/CD24^{-/low} [97]. With a novel technique for the detection

and characterization of secreted proteins from viable tumor cells, the EPISPOT assay, it was recently shown that both BM from metastatic and primary breast cancer patients contain DTCs with a breast cancer stem cell-like phenotype [41] characterized by cytokeratin 19 positivity and absence of Muc-1 secretion [98]. Further, recently described characteristics of DTCs are also consistent with their putative stem cell phenotype. Thus, most DTCs are in a nonproliferative state that renders them resistant to systemic chemotherapy and allows long-term persistence [18]. Abrogation of tumor cell dormancy in DTCs, the presence of which has prognostic relevance [19], by genetic aberrations or changes in the microenvironment might contribute to metastatic relapse.

Conclusion

Detection of DTCs/CTCs according to standardized protocols and subsequent comprehensive phenotypical and molecular characterization of these cells might contribute to an improved identification of patients in need of additional systemic anticancer therapy, to the stratification of patients to adjuvant therapies (for example Herceptin) and finally to the development of more tailored and personalized therapies for breast cancer patients. However, only prospective trials will show whether individual DTCs and CTCs are representative for the behavior of the entire pool of occult tumor cells and drug sensitivities of the corresponding cancer tissue. Furthermore, the impact of DTCs/CTCs as surrogate marker to monitor therapeutic interventions remains to be elucidated in future studies.

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