

Circulating Tumor Cells in Early-Stage Breast Cancer

Klinische Relevanz von zirkulierenden Tumorzellen beim Mammakarzinom

Authors

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Key words

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- biomarkers

Abstract

Disseminated tumor cells (DTC) are routinely detected in bone marrow (BM) in 30–40% of primary breast cancer patients. Positive BM status at the time of diagnosis as well as DTC persistence after therapy are strong independent prognostic factors. Since repeated BM aspirations are not well tolerated, detection of single tumor cells in peripheral blood (circulating tumor cells; CTC) have become of interest in recent years. CTC are found in 10–80% breast cancer patients. Variability can be explained by stage of the disease and detection method. Emerging data have shown CTC to be of prognostic relevance for both, patients with primary and metastatic disease. The assessment of CTC in blood may become an important biomarker for prognostication and therapy monitoring. Determination of their molecular characteristics will enable specific targeting of minimal residual as well as metastatic disease. This review summarizes recent research and future perspectives.

Zusammenfassung

Disseminierte Tumorzellen im Knochenmark können in 30–40% aller Patientinnen mit primärem Mammakarzinom entdeckt werden. Der Nachweis von disseminierten Tumorzellen zum Zeitpunkt der Erstdiagnose, aber auch die Persistenz derselben unter einer Therapie sind ungünstige prognostische Faktoren. Die Praktikabilität von wiederholten Knochenmarkspunktionen unter einer Therapie ist jedoch zeit- und kostenintensiv und für die Patientin unangenehm. Deswegen bietet die Untersuchung von peripherem Blut deutliche Vorteile. Zirkulierende Tumorzellen können in 10–80% aller Patientinnen entdeckt werden. Die Rate hängt von der Analyse-methode und dem Stadium der Erkrankung ab. Es konnte bereits nachgewiesen werden, dass das Vorhandensein von Tumorzellen im peripheren Blut sowohl für Patientinnen mit Metastasen als auch für Patientinnen ohne Metastasen von prognostischer Bedeutung ist. Eine molekulare Charakterisierung der zirkulierenden Tumorzellen könnte weiterhelfen, spezifische Therapien zu entwickeln, die sich direkt gegen diese Tumorzellen richten und so helfen, diese zu eliminieren. Dieser Übersichtsartikel fasst die aktuelle Literatur zusammen und gibt eine Zukunftsperspektive zu dem Thema.

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Abbreviations

BM	bone marrow
CTC	circulating tumor cell
DFS	disease-free survival
DTC	disseminated tumor cell
EFS	event-free survival

EpCAM	epithelial cell adhesion molecule
ICC	immunocytochemistry
HER2	human epidermal growth factor receptor 2
MRD	minimal residual disease
OS	overall survival

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Introduction

▼ The theory on hematogenous cancer spread was introduced by several authors in the 19th century [1]. Paget's "seed and soil" hypothesis emphasized the complex interactions between tumor cells and the microenvironment of the homing body sites [2]. In the last two decades various methods have been established to detect and characterize single tumor cells in bone marrow (BM) and blood, and clinical trials have been conducted to assess their clinical usefulness.

The presence of disseminated tumor cells (DTC) in BM is a common phenomenon in solid tumors and can be observed in 30–40% of primary breast cancer patients [3–6]. As demonstrated by a large analysis of specimens from over 4700 patients, detection of DTC at the time of diagnosis is associated with poor clinical outcome with respect to disease-free survival (DFS) and overall survival (OS) at a level of evidence of I [6]. Persistence of DTC after completion of surgical and systemic therapy correlates with decreased median survival as well [7].

However, one major limitation of DTC detection is the invasive character of bone marrow aspiration. Since BM punctures are not well tolerated by many patients, recent research has focused increasingly on the assessment of circulating tumor cells (CTC) in peripheral blood. Obviously, detection of CTC is easier and more feasible for repeated analysis than identification of DTC in BM. Detection rates in the blood vary, depending on method and stage of the disease, between 10–40% in primary breast cancer and are significantly higher in patients with metastatic disease ranging from 40 to 80%. While the biological significance of DTC is generally accepted, prognostic relevance of CTC detection remains yet to be conclusively cleared. However, recent data support major prognostic potential of CTC in both patients with primary and metastatic breast cancer [8–10].

Further, there is urgent need to evaluate new markers for prediction of therapy response. CTC assessment may thus serve as an important biomarker for prognostication, prediction and therapy monitoring, and its pheno- and genotyping have a potential to enable targeting of minimal residual disease [11]. In the following review we will discuss the role of CTC as a novel diagnostic tool in early-stage breast cancer.

Methods for Detection and Characterization of CTC

▼ At present, two main approaches are in use for the detection of single tumor cells: antibody-based (using antibodies against epithelial markers, e.g. cytokeratin) and molecular assays (based on amplification of epithelial-specific mRNA) [12, 13]. The low frequency of CTC in the blood explains the need for sensitive detection methods and efficient enrichment techniques. Automated equipment for identifying and analyzing CTC has been and is continuously developed [13]. Moreover, some study groups apply their own cut-off value to determine a sample "positive" [14]. Recently, commercially available standardized diagnostic approaches, particularly CellSearch® (Veridex, Warren, NJ, USA) and AdnaTest (AdnaGen AG, Langenhagen, Germany), have been established and incorporated into translational research programs within large clinical trials. The CellSearch® system is a semiautomated antibody-based assay based on immunofluorescence and flow cytometry [8, 14]. After the initial enrichment step using immunomagnetic beads linked with antibodies against the cell surface protein EpCAM (Epithelial cell adhesion

molecule) tumor cells are identified and quantified by cytokeratin-positivity, positive nuclear staining and CD45 negativity. With regard to sensitivity and specificity of the CellSearch® system, defining a distinct number of positive cells/ml blood for CTC-positivity is essential. However, different cut-off values were used within clinical trials evaluating the impact of CTCs in primary breast cancer patients. There is evidence that the total number of CTCs is meaningful [9], but further studies are needed for standardization. RT-PCR based AdnaTest BreastCancer enriches CTCs during the first step by immunomagnetic beads labeled with antibodies against MUC1 and EpCAM. In the following step, mRNA is extracted from captured epithelial cells, cDNA is reversely transcribed and amplicons for GA 73.3, EpCAM, and HER2 are amplified by multiplex PCR [12, 15, 16]. The concordance rate between both systems is high reaching 70–90% [12]. For a standardization of CTC measurements, diagnostic assays are still to be optimized with regards to reproducibility, enumeration and molecular characterization of CTCs. This can be achieved for example by minimizing cell loss and preserving cell morphology. Therefore a prospective multicenter study was recently initiated to compare different assays for CTC detection and characterization (www.detect-study.de).

Correlation of CTC and DTC Detection

▼ Bone marrow, as a common homing organ for tumor cells of epithelial origin, has traditionally been the main compartment in which the prognostic impact of detected tumor cells has been pursued. While the prognostic value of DTC is well-established [6], this method has a disadvantage that the BM is collected by an invasive procedure. It is therefore not suitable for repeated sampling during e.g. therapy monitoring. The association between the presence of CTC and DTC is hard to determine. So far, only limited data are available on the comparison of isolated tumor cells in blood and in BM, and studies have resulted in partly contradictory data. CTC incidence varies due to methodological differences and few reports investigated the presence of tumor cells in both compartments within the same patient group.

Nevertheless, the CTC counts are generally lower than the number of DTC. Several authors reported a high percentage of patients positive for DTC who remain CTC negative [17–21]. In a study conducted by our group tumor cell detection significantly correlated in both compartments [22]. Possibly isolated tumor cells in BM represent a robust cell population with high recovery rates after enrichment and detection techniques. Accordingly, presence of tumor cells in peripheral blood may not reflect minimal residual disease as well as BM positivity, as blood is by many considered to be only a temporary compartment for tumor cells while BM acts as a "filter" for tumor cells [23]. Further, the exact mechanism of tumor cell release into the bloodstream is poorly understood; possibly single cells are shed not constantly, but intermittently. This may result in false-negative detection rates if a single-point sampling is evaluated.

Prognostic Information from CTC Detection in Primary Breast Cancer Patients

▼ The prognostic impact of DTC presence in bone marrow at diagnosis was confirmed in a large pooled analysis [6]. Peripheral blood analyses are more acceptable to the patients; therefore

translational research has incorporated CTC detection into large clinical trials. Hematogenous tumor cell spread occurs at a very early stage of the disease, long before the primary tumor becomes clinically detectable – in 10–40% of patients with early-stage breast cancer and no evidence of distant metastasis CTC are routinely detected. Prognostic relevance of CTC in metastatic setting where 40–80% of metastatic breast cancer patients are CTC-positive has been demonstrated in numerous trials [8, 14, 24–30]. In contrast, clinical implications of CTC detection in early-stage breast cancer are still under investigation.

As part of the translational research program of the SUCCESS-trial (www.success-studie.de), peripheral blood samples from 2000 lymph node-positive and/or high risk lymph node-negative breast cancer patients before and after adjuvant anthracycline and taxane containing systemic therapy were evaluated for the presence of CTC using the CellSearch® system. 435 (22%) of patients presented with at least one CTC at diagnosis. During the median 35-month follow-up, 114 (6%) women recurred and 66 (3%) died of breast cancer. In women with one or more CTC, disease-free survival at three years was 88.1% compared with 93.7% in CTC-negative women ($p < 0.0001$). The detection of CTC before treatment was confirmed in multivariate analysis as independent predictor for both DFS (HR = 1.88) and OS (HR = 1.91) [31]. Similar impact on clinical outcome was reported previously by smaller studies (Table 1).

Detection of Persistent CTC after Completion of Therapy

Previous studies have shown that chemotherapy is not effective in complete eradication of DTC from bone marrow [38, 39]. According to data presented by Janni et al., the persistence of DTC is an independent predictor of increased relapse risk [7, 40]. Data on prognostic impact of persistent CTC from the SUCCESS-trial were presented at the 2010 ASCO Annual Meeting [9]. More than 1 CTC was detected in 9% of patients after completion of adjuvant cytotoxic therapy; these patients had a significantly shorter disease-free interval while overall survival remained unaffected. Detection of > 5 CTC was a significant indicator of worse prognosis for both DFS and OS.

The “GeparQuattro” trial is to date the largest evaluation of CTC in the context of neoadjuvant therapy (www.germanbreastgroup.de/geparquattro). CTC detection was assessed before and after neoadjuvant cytotoxic therapy. 22% patients had at least one CTC before treatment; the positivity rate decreased to 11% after

chemotherapy [41]. However, the pathological response of primary tumor was not reflected by CTC changes. No correlation between primary tumor response to therapy and CTC detection was observed in another neoadjuvant phase-II trial by Pierga et al.: the presence of persistent CTC after therapy significantly predicted reduced relapse-free survival [34]. In contrast, another study identified decreased CTC counts in patients whose tumor responded to therapy favorably [42]. A poor response of CTC correlated with early distant relapse. These data suggest that monitoring the response of CTC to therapy provides information on therapy success and that persistent minimal residual disease is clinically relevant and may have a different chemosensitivity than the primary tumor. However, the question whether patients with persistent CTC benefit from intensified or longer systemic therapy remains as yet unclear. Therefore, whether CTC detection can improve management of early-stage breast cancer patients can only be answered through a large prospective trial.

Geno- and Phenotyping of CTC

Single tumor cells in secondary “homing sites” are assumed to be a surrogate marker for minimal residual disease (MRD). Beyond mere detection of CTC, their characterization is aimed to identify relevant features for targeted therapy. All adjuvant strategies are developed to eradicate minimal residual disease. However, treatment decisions regarding endocrine or HER2-targeted therapy are based on the pheno- and genotype of primary tumor cells. While the local and regional therapy is suitable to manage primary tumor and local lymph nodes, the success of systemic therapy depends on its ability to eradicate occult tumor cells before they become clinically apparent [43]. Available data suggest a more complex relationship between the primary tumor and DTC/CTC, with considerable discrepancies at the genomic level [44]. As reported previously, DTC and CTC can exhibit features different from those of the primary tumor, especially with respect to ER and HER2 status [12, 22, 45–47]. The majority of these cells persist in a non-proliferating “dormant” state characterized by downregulated expression of the proliferation marker [48]. This dormant state might explain the reduced efficacy of adjuvant chemotherapy in eradicating MRD as most cytotoxic therapies target proliferating cells [49].

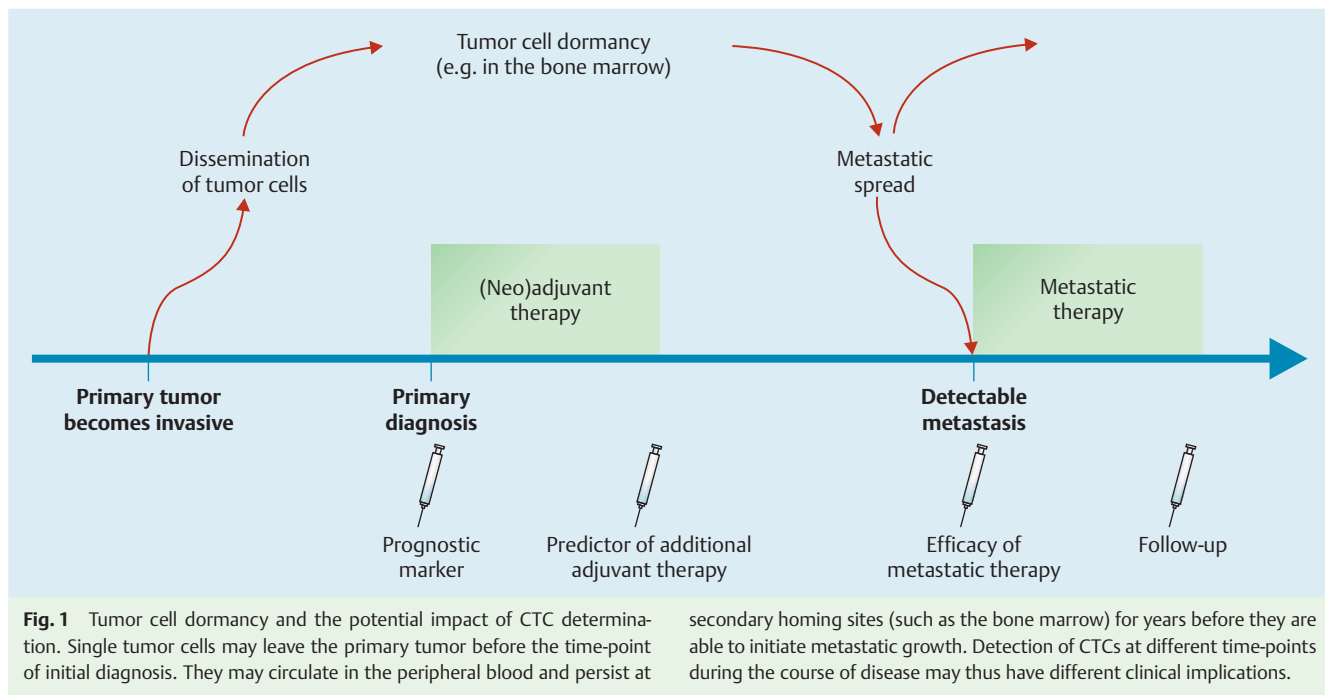
Isolated tumor cells in bone marrow and blood represent a heterogeneous population with regard to the expression of steroid hormone receptors, adhesion molecules, growth factor receptors, major histocompatibility complex antigens etc. Generally, DTC/

Table 1 Prognostic significance of CTC detection in primary breast cancer patients.

Author	Year	Number of patients	Method	Positivity rate (%)	Follow-up (months)	Prognostic relevance
Rack [31]	2010	2 026	CellSearch®	22% ¹	35	DFS, OS
Rack [9]	2010	1 489	CellSearch®	9% ⁴	32	DFS ² , OS ¹
Bidard [32]	2010	115	CellSearch®	23%	36	DFS, OS
Daskalaki [33]	2009	165	RT-PCR	55% ¹ , 52% ²	59	OS ¹
Pierga [34]	2008	118	CellSearch®	23% ¹ , 17% ²	18	DFS ³
Xenidis [35]	2006	167	RT-PCR	22%	32	DFS, OS
Ntoulia [36]	2006	101	RT-PCR	14%	24	DFS
Nieto [28]	2004	242	ICC	7%	84	EFS, OS
Stathopoulou [37]	2002	148	RT-PCR	30%	28	DFS, OS

DFS: disease free survival; OS: overall survival; EFS: event free survival; RT-PCR: reverse transcriptase polymerase chain reaction; ICC: immunocytochemistry; ¹ before chemotherapy;

² after chemotherapy; ³ combined positivity before and/or after neoadjuvant chemotherapy; ⁴ at least one CTC.



secondary homing sites (such as the bone marrow) for years before they are able to initiate metastatic growth. Detection of CTCs at different time-points during the course of disease may thus have different clinical implications.

CTC frequently feature factors linked with poor clinical outcome, e.g. negative hormone receptor status and up-regulation of urokinase-type plasminogen activator receptor. The epidermal growth factor receptor HER2 is of particular interest. Its expression is highly predictive of response to trastuzumab therapy [50]. Interestingly, HER2 gene amplification can be acquired during disease progression; patients with initially HER2-negative primary tumor may be diagnosed with HER2 overexpressing CTC [51]. Despite this observation patients with HER2-negative primary tumors are not eligible for HER2-targeted therapy regardless of HER2-status of MRD. Recent data suggest that evaluation of the HER2-status of CTC/DTC may identify additional patients who can benefit from HER2-targeted therapy [52]. Rack et al. presented results of a small interventional post-adjuvant trastuzumab-based pilot study [53]: all patients were recurrence-free and asymptomatic and presented with persistent HER2-positive DTC; in these patients trastuzumab therapy eradicated HER2-positive DTC. Similar results were previously reported by Bozionellou et al. [54]. HER2-targeted therapy effectively eliminated HER2-positive MRD in 90–95% initially DTC/CTC-positive patients. Whether effective eradication of tumor cells in secondary homing sites favorably affects clinical outcome, remains to be cleared in large prospective randomized trials.

With respect to ER status, we reported previously a striking discrepancy between the primary tumor's status and that of disseminated tumor cells [47]. CTC are mostly hormone receptor negative despite an ER-positive tumor [22, 55]. One possible explanation is the noted heterogeneity of the primary tumor; ER-negative tumor cells may have a survival advantage due to their more aggressive phenotype and are therefore more likely to disseminate. Inversely, ER-positive breast cancer cells are known for their decreased invasiveness and metastatic potential [47]. Considering natural history of breast cancer progression, it has been reported that up to 30% of patients with ER-positive tumors develop ER-negative metastases [56]. These observations may be relevant to clinicians when selecting patients for endocrine therapy,

as we assume that ER-negative CTC/DTC would not respond to such therapy [57]; in such cases the loss of ER-positivity in MRD may explain the failure of endocrine therapy. The ability to assess features of MRD and to follow changes in their pheno- and genotype during and after treatment, may prospectively allow more individual therapy.

Phenomenon of Tumor Cell Dormancy

Tumor cell dormancy commonly describes long latency intervals of cancer growth, lasting from completion of primary therapy to the clinical diagnosis of relapse (● Fig. 1). Based on epidemiological studies, breast cancer recurrence can occur after a very long time interval, sometimes a decade or more [58]. On the cellular level, isolated tumor cells in secondary homing sites, such as bone marrow and blood, may persist in a non-proliferative inactive ("dormant") state for many years [39]. Meng et al. examined blood samples from 36 dormancy candidates, i.e. asymptomatic women with no evidence of disease 7 to 22 years following mastectomy. In thirteen cases CTC were detected [23]. However, tumorigenic potential of these cells seems limited. Molecular mechanisms of tumor cell dormancy are not yet well understood. Dormant cells show very low proliferation levels [48] and probably alternate between phases of active and arrested growth. Proliferation appears to be counterbalanced by cell death in these patients, holding MRD in a steady-state. It is unclear, which factors in the cells or their microenvironment disturb this balance to eventually stimulate metastasis and cancer growth. Since cytotoxic therapies mostly target rapidly proliferating cells, low level proliferation of dormant cells may explain why they successfully elude such treatment [9, 43]. Because of its reported chemoresistance targeted approaches and bisphosphonate therapy have gained more interest in treating MRD [11, 59, 60]. Which tumor cells are potential candidates for tumor cell dormancy, is not clear. According to the metastatic inefficiency mod-

el, only a very small percentage of tumor cells is able to persist after leaving the primary tumor. The metastatic cascade consists of a series of steps; failure in any one of these steps leads to elimination of tumor cells [61,62]. Large numbers of primary tumor cells are shed into blood circulation daily and need to survive in the blood stream until they can arrest in a new homing site. The microenvironment in this secondary site is supposed to change the gene-expression patterns of tumor cells and therefore affect their growth ability. 99.9% of shed cells are believed to perish during the dissemination process resulting in an oligoclonal seeding of distant (micro)metastases. This “metastatic inefficiency” is consistent with tumor cell studies since the majority of CTC/DTC-positive patients will not suffer from a relapse despite their positive BM or blood status [6].

Cancer Stem Cell Theory

Recent studies on stem cell biology have given new impetus to the cancer stem cell theory. This conceptual model holds that tumors may originate from a small subclone of cells with stem cell properties [63].

Consequently, tumors contain a small cell fraction with stem cell properties. In contrary to the traditional model of cancer growth, which postulates that oncogenesis is caused by random mutations of oncogenes and tumor suppressors which equally affect all cells. The stem cell model assigns the potential to proliferate and to give rise to secondary tumors to a rare subpopulation of cells (“cancer stem cells”). These highly tumorigenic cells are able to reinitiate tumor growth even after removal of the primary tumor and completion of systemic therapy [63].

Experimental data suggest an important role of cancer stem cells in development and progression of various tumor entities, such as breast and gastrointestinal cancer, retinoblastoma and ovarian cancer [64,65]. For instance, Balic et al. reported that the majority of DTC have a putative cancer stem cell phenotype, such as ALDH1 positivity or the presence of CD44 and absence of CD24 [63,66]. However, these characteristics were not measured on the same DTC in a multiplexed fashion. Such features may provide efficient DNA repair capacity, inherent resistance to apoptosis, asymmetric division and clonal expansion and make cells resistant to therapy [67]. The study of Balic et al. supports the provocative theory that DTC from the bone marrow of early-stage breast cancer patients represent in fact tumor initiating cells, and suggests that these tumor cells display biological properties that enable their spread and subsequent colonization of distant sites.

Conclusions

Circulating tumor cells may become a useful tool for prognostication and therapy monitoring and an important step towards more individualized therapy in early-stage breast cancer. The implications regarding choice of adjuvant therapy can only be answered in randomized clinical trials stratifying patients based on expression profiles of minimal residual disease rather than primary tumor's.

Conflict of Interest

None.

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