

## Circulating Tumor Cells and Bone Marrow Micrometastasis

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**Abstract** Sensitive immunocytochemical and molecular assays allow the detection of single circulating tumor cells (CTC) in the peripheral blood and disseminated tumor cells (DTC) in the bone marrow as a common and easily accessible homing organ for cells released by epithelial tumors of various origins. The results obtained thus far have provided direct evidence that tumor cell dissemination starts already early during tumor development and progression. Tumor cells are frequently detected in the blood and bone marrow of cancer patients without clinical or even histopathologic signs of metastasis. The detection of DTC and CTC yields important prognostic information and might help to tailor systemic therapies to the individual needs of a cancer patient. In the present review, we provide a critical review of (a) the current methods used for detection of CTC/DTC and (b) data on the molecular characterization of CTC/DTC with a particular emphasis on tumor dormancy, cancer stem cell theory, and novel targets for biological therapies; and we pinpoint to (c) critical issues that need to be addressed to establish CTC/DTC measurements in clinical practice.

Metastasis is the main cause of death in patients with solid epithelial tumors (i.e., carcinomas), which represent the majority of cancers in industrialized countries. Recently, Bernards and Weinberg (1) presented a new metastasis model in which the metastatic capacity is gained early during primary tumor development. Indeed, disseminated tumor cells (DTC) can be already detected at early stages of tumor progression in regional lymph nodes, peripheral blood, and in bone marrow of cancer patients using highly sensitive detection methods (2). Interestingly, bone marrow has emerged as a common homing organ for metastatic epithelial tumor cells, independent of the primary tumor site and the pattern of overt metastases (Table 1).

Here, we will (a) review the methods currently used for the detection of circulating tumor cells (CTC) in the peripheral blood and DTC in the bone marrow, (b) point out the most critical issues in interpreting the findings obtained with these methods, and (c) describe the molecular characteristics of CTC/DTC with an emphasis on potential therapeutic targets.

### CTC/DTC Detection Methods: Potential and Limitations

The unambiguous identification and characterization of CTC/DTC requires extremely sensitive and specific analytic

methods. Detection methods are usually used in combination with tumor cell enrichment procedures, including density gradient centrifugation (Ficoll-Hypaque separation), immunomagnetic procedures, or size filtration methods to enrich tumor cells before their detection (3–6). Here, we will focus on the current CTC/DTC detection methods (Fig. 1; Table 2).

**Immunologic techniques.** One major approach to identify CTC/DTC is immunocytochemical staining with monoclonal antibodies against epithelial or tumor-associated antigens (7–9). The prognostic effect of immunocytochemical DTC analysis done at the time of primary surgery was confirmed in a recent large pooled analysis including 4,703 breast cancer patients with a 10-year follow-up (10). However, additional studies are required to determine whether the DTC analysis will help to change the clinical management of the cancer patient. To date, cytokeratins have become the most widely accepted protein markers for the detection of epithelial tumor cells in mesenchymal tissues such as bone marrow, blood, or lymph nodes (9–12). However, loss of cytokeratin expression can occur in cancer cells (13), which may result in false-negative results depending on the spectrum of cytokeratin proteins recognized by the antibody used. Moreover, different staining techniques can result in specificity variations (14, 15). Several international organizations have therefore recognized the need for standardization of the immunocytochemical assay and for its evaluation in prospective studies (9, 16), and the search for better CTC/DTC markers is still ongoing.<sup>3</sup>

The use of new automated devices for the microscopic screening of large amounts of immunostained slides has already helped to increase speed and reproducibility of immunocytochemical analyses (17–22). Among the commercially available semiautomated approaches, the CellSearch system, which has been Food and Drug Administration approved for monitoring of blood from metastatic breast and colon cancer patients, has gained considerable attention because it allows both automated

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**Table 1.** Detection rate of DTC in bone marrow of patients without distant overt metastases (stage M<sub>0</sub>)

Tumor type	Detection rate (%)	References
Breast cancer	20-40	(10)
Prostate cancer	20-50	(113-116)
Lung cancer (NSCLC)	40-60	(117-119)
Gastric cancer	35-60	(120, 121)
Esophageal cancer	30-40	(60, 122)
Pancreatic cancer	20-35	(112, 123, 124)
Colorectal cancer	20-30	(123, 125, 126)
Head and neck cancer	20-30	(127-131)

Abbreviation: NSCLC, non-small cell lung cancer.

immunomagnetic epithelial cell adhesion molecule-based (EpCAM) enrichment as well as cytokeratin staining of CTC in blood samples (23-25). This standardized method was recently shown to be associated with a high intraobserver and interobserver as well as interinstrument accordance (24, 25). Although cells fulfilling the criteria of CTC were very rarely found in healthy women and patients with benign diseases, CTC could be detected in peripheral blood samples of patients with metastatic disease from all major carcinomas (24). Moreover, the detection of 5 or more CTC/7.5 mL blood in 43 of 83 (52%) metastatic breast cancer patients before first-line chemotherapy was highly predictive for progression-free and overall survival (26) and

provided more helpful information than conventional imaging procedures (27).

Very recently, a new microchip technology using a microfluidic device ("CTC-chip") was reported, allowing isolation of CTC from blood of cancer patients (28). This CTC-chip consists of an array of 78,000 microposts that are coated with anti-EpCAM antibodies. The selection of target CTC is mediated by the interaction of these cells with the EpCAM-coated microposts under precisely controlled laminar flow conditions. The CTC-chip detected cytokeratin<sup>+</sup>-CTC in nearly all patients (115 of 116) with lung, prostate, pancreatic, breast, and colon cancer. Surprisingly, CTC detection rates and CTC numbers were independent from disease stage (28), and future studies on the clinical utility of this interesting technology are needed.

Another approach is the laser scanning cytometer, which combines the speed of the flow cytometry with the power to analyze a single positive event for its morphologic properties (29) and first clinical data are promising (30). This technology combined a RBC lysis with EpCAM-based immunostaining and revealed between 50 and 300,000 circulating epithelial cells per milliliter of blood in >90% of cancer patients (29). The number of events detected is 2 to 3 log units higher than those obtained with most other CTC detection technologies and this may warrant further studies on the specificity of this approach.

Another new technique that allows the detection of only viable cells after a CD45<sup>+</sup> cell depletion was introduced for DTC/CTC analyses from bone marrow aspirates and blood samples (31). This technique was designated EPISPOT

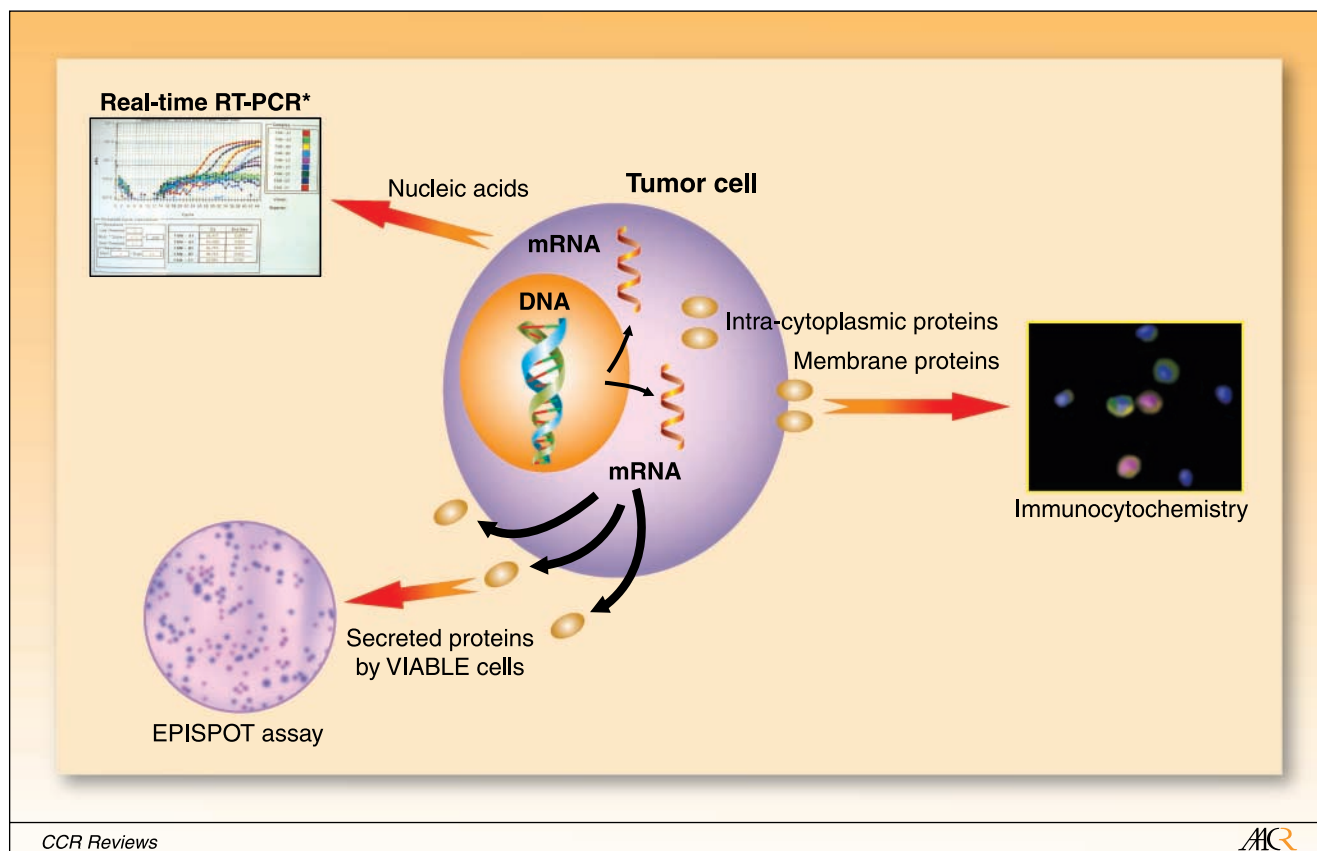


Fig. 1. Schematic view of CTC/DTC detected by sensitive current methods.

**Table 2.** Summary of the advantages and disadvantages of CTC/DTC detection methods

Detection methods	Advantages	Disadvantages
Immunocytochemical methods	Morphological analysis of CTC/DTC Multiple labeling of antigens on CTC/DTC Direct quantification of CTC/DTC Isolation of CTC/DTC for further analyses	Subjective analyses for CTC/DTC identification Time-consuming screening of tumor cells
RT-PCR	High sensitivity Observer-independent detection of CTC/DTC Quantitative real-time RT-PCR: higher specificity	Deficient expression of target mRNAs in CTC/DTC RNA instability Low-level illegitimate expression in normal cells No visualization and isolation of CTC/DTC possible
EPISPOT assay	High sensitivity Detection of viable CTC/DTC Detection of secreted proteins	Proteins must be actively secreted, shed, or released No identification and isolation of secreting cell possible

(EPithelial ImmunoSPOT), a protein-secreting profiling based on the secretion or active release of specific marker proteins using an adaptation of the enzyme-linked immunospot technology (Fig. 2). As immunospots are the protein fingerprint left only by the viable releasing epithelial cells, a cell culture is needed to accumulate a sufficient amount of the released marker proteins. Thus, dying cells that do not secrete adequate amounts of protein are not detected (32, 33). Besides the mere detection of CTC/DTC, this assay can provide important information on the profile of secreted proteins potentially relevant for metastasis formation (34).

In conclusion, the clinical utility of all of these new approaches need to be validated in large-scale studies on cancer patients.

**PCR-based assays.** PCR-based assays have become the most widely used alternative to immunocytochemical assays. Although DNA markers (e.g., *k-ras* mutations) have been previously used by some groups in colon and pancreatic cancer (7), most groups use reverse transcription-PCR (RT-PCR) assays targeting specific RNA markers. Many transcripts have been evaluated as "tumor-specific" markers like *CK18*, *CK19*, *CK20*, *Mucin-1*, and *carcinoembryonic antigen* (35–40). However, these transcripts can also be identified by sensitive RT-PCR assays in normal bone marrow, blood, and lymph node tissue (41–43), and clear cutoff values need to be defined and validated in multicenter trials.

There is a plethora of single-institution data on the prognostic significance of data obtained with the RT-PCR approach in various tumor entities (3), whereas large-scale multicenter validation studies are still missing. In breast cancer, the detection of *CK19* mRNA transcripts in blood samples correlated with adverse survival outcome (44), in particular in early-stage patients with estrogen receptor–negative tumors. However, expression of *CK19* mRNA marker can be down-regulated (13), which argues in favor of a multimarker RT-PCR approach.

### Molecular and Functional Characterization of DTC

**Genomic characteristics and gene expression profiles.** Comparative genomic hybridization showed that DTC in the bone marrow are genetically heterogeneous in early-stage breast cancer patients with fewer genetic aberrations than in advanced stages (45, 46). Moreover, these cells lack genomic aberrations observed in arbitrary selected areas of the primary tumors (47).

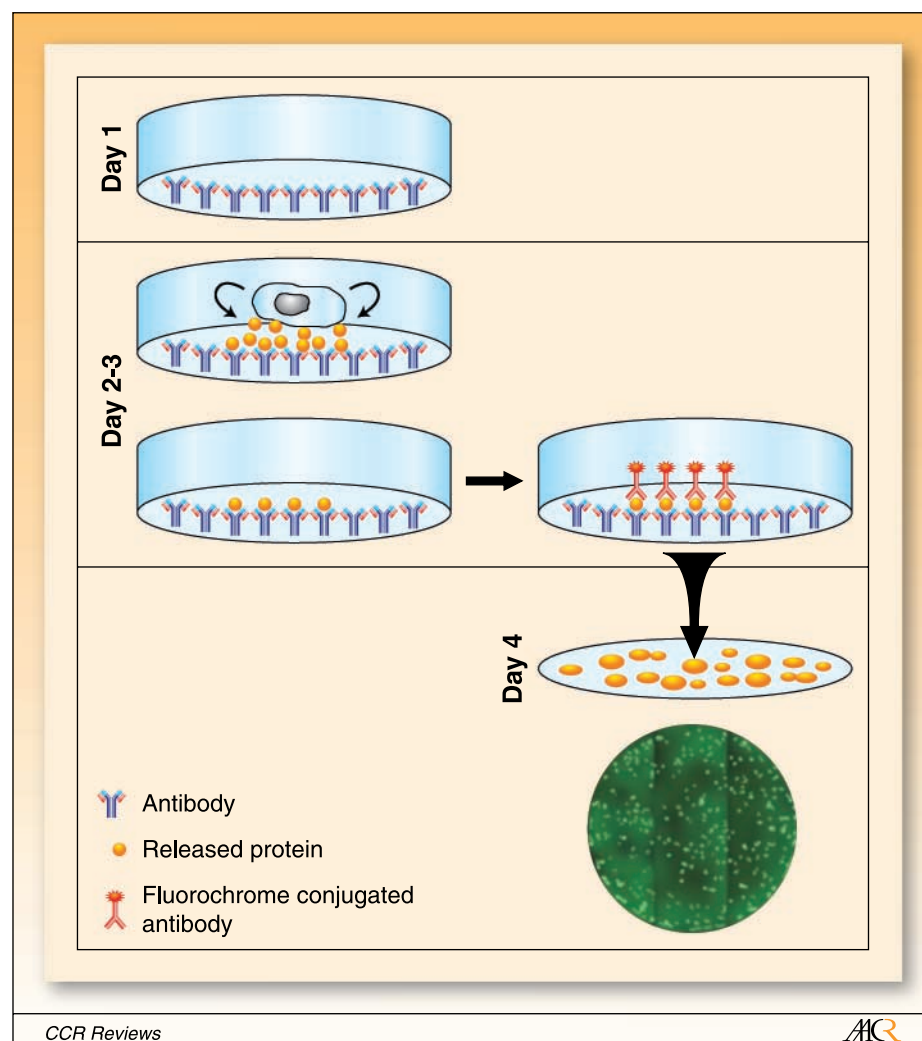
Thus, DTC may evolve independently from the primary tumor and accumulate more genomic aberrations after their homing in the bone marrow and other distant organs (48). Recent data indicate that CTC in prostate cancer patients are derived from distinct tumor foci, which usually represent the hormone-refractory tumor fraction (49) that can now be treated by new targeted therapies, e.g., against insulin-like growth factor receptor-1.

Microarray-based expression analyses on primary breast tumors revealed specific gene signatures in patients with DTC in bone marrow (50, 51). These findings support the concept that tumor cells acquire the genetic changes relevant to their metastatic capacity early in tumorigenesis (1) and that the metastatic potential is therefore encoded in the bulk of primary tumor cells (1, 52).

Thus far, there is only limited information about the global gene expression program in DTC and CTC. Smirnov et al. (53) generated gene expression profiles for CTC enriched from blood of a small number of metastatic carcinoma patients and showed that gene expression profiles of CTC may be used to distinguish advanced cancer patients from normal donors. In a study reported by Watson et al. (54), DTC in the bone marrow after chemotherapy possessed unique transcriptional signatures. One of the transcripts up-regulated in DTC was *TWIST 1*, a transcription factor that previously had been identified to play an important role in metastasis by promoting epithelial-mesenchymal transition (55–58).

**Homing, dormancy, and growth of disseminating tumor cells.** The properties of primary tumor cells that enable them to lodge into bone marrow are still under investigation. Tumor cells, for example, express the CXC-chemokine receptor CXCR4 at their membrane and metastatic cells may use this chemokine-mediated mechanism to home at bone marrow, which is rich in SDF-1, the chemokine binding to CXCR4 (59). Expression of CXCR4 in primary human tumors (e.g., esophageal cancer) is related to the presence of DTC in lymph nodes and bone marrow (60), indicating that the CXCR4-SDF1 interaction may play a role in early tumor cell dissemination.

After homing, DTC need to express/secrete relevant proteins to form a solid metastasis. Epidermal growth factor and fibroblast growth factor-2—two known stem cell growth factors—were relevant for the *in vitro* growth of DTC obtained from bone marrow of cancer patients and the growth capacity of these cells was correlated to the clinical outcome of the



**Fig. 2.** EPISPOT assay procedure. Day 1, the membranes of the ELISPOT plates are coated with a specific antibody. Days 2 to 3, the cells are seeded in each well and cultured for 48 h. During this incubation period, the released specific proteins are directly immunocaptured by the immobilized antibody on the bottom of the well. Plates are then washed and cells were removed. The presence of the released protein is revealed by the addition of a fluorochrome-conjugated antibody. Day 4, fluorescent immunospots are counted with an automated reader. One immunospot corresponds to the fingerprint left only by one viable cell releasing the marker protein.

respective patients (61). Interestingly, a subset of prostate CTC can secrete fibroblast growth factor-2 (34). Future clinical follow-up studies will show whether patients with these particular cells might have an increased risk to develop metastatic relapse.

Only half of the breast cancer patients had DTC relapse, whereas the other half remained free of overt metastasis over a 10-year follow-up period (10). Thus, a significant fraction of DTC might never develop into overt metastases but remain in a "dormant" state. However, the persistence of DTC in bone marrow even years after primary treatment is linked to an increased risk of late metastatic relapses in breast cancer (62, 63). Thus far, little is known about the dormant cells and the conditions required—environmental factors (e.g., stress, immune system, dietary changes)—for their "awakening" from the dormant or quiescent phase into the dynamic phase of metastasis formation (64). The steady-state regulating dormancy might be disturbed by both changes in the DTC (e.g., additional mutations) and the surrounding microenvironment (e.g., decrease in immune surveillance or increased angiogenic potential; refs. 65, 66). Among the protein characteristics, expression of the tyrosine kinase receptor HER2 and the urokinase-type plasminogen activator receptor

on CTC or DTC are correlated to metastatic relapse in breast cancer and gastric cancer, respectively (67–69). Thus, signaling mediated by Her2 and urokinase-type plasminogen activator receptor might be important for the transition of DTC from a dormant stage to an active growth phase, and future strategies aimed at inducing and/or maintaining tumor cell dormancy may include concomitantly blocking of these proteins (70).

Recent data by Kent Hunter's group have opened the interesting possibility that metastatic efficiency might be modulated by polymorphisms that are present on the heterogeneous genome of the human population (71). Ongoing studies will show whether these exciting data obtained in animal models can be extrapolated to humans.

**In search of putative metastatic stem cells.** Recently, the search for breast cancer stem cells has gained an increasing attention with the discovery of new stem cell markers and signatures (34, 72–74). The cancer stem cell concept hypothesizes that tumors arise from a small subpopulation of stem cells and it has been assumed that the founder cells of overt metastases might be also stem cells disseminated from the primary tumor to the distant metastatic site (75). This assumption is supported by the observation that primary tumor stem cells show an expression profile associated with



metastatic relapse in breast cancer patients (74). Moreover, Ginestier et al. (76) showed that the expression of a new breast stem cell marker, ALDH1, was associated with poor clinical outcome in breast cancer patients.

The significant correlation between the presence of DTC in bone marrow and metastatic relapse (10) suggests that the founder cells of overt metastases might be among those DTC as metastatic stem cells. Furthermore, most CTC/DTC are non-proliferating (i.e., Ki-67 negative) and resistant to chemotherapy (77–80), as postulated for cancer stem cells. Moreover, the study of Balic et al. (81) represents an important first step in showing that micrometastases isolated from the bone marrow 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 DTC in bone marrow have a putative breast cancer stem cell phenotype. It also suggests that these cells may display biological properties that facilitate their metastatic spread, enabling them to colonize distant sites: (a) increased angiogenic capacity and (b) express receptors, such as CXCR4 (82). In addition, a subpopulation of viable DTC that are  $CK19^+MUC1^-$  has also been previously suggested as breast stem cell-like cells (34). Thus far, it is, however, still unclear whether DTC have self-renewal ability, the hallmark of stem cells.

**Identification of therapeutic targets.** CTC/DTC can show properties distinct from the primary tumor and the characterization of these cells could therefore help to select cancer patients for targeted therapies. In breast cancer, the *HER2* oncogene has become the most prominent target for biological therapy with a humanized anti-*HER2* monoclonal antibody (trastuzumab; refs. 83, 84). Currently, all patients are stratified to this targeted therapy by primary tumor analysis only. However, recent reports have shown that *HER2*-positive DTC and CTC can also be detected in patients with *HER2*-negative primary tumors (67, 85, 86). These findings are consistent with our previous data on the high frequency and prognostic relevance of *HER2* expression on DTC in bone marrow (87), suggesting that additional patients could benefit from *HER2*-directed therapies (86). Ongoing clinical studies will reveal whether the *HER2* status of DTC or CTC may predict response to trastuzumab or other *HER2*-directed therapies.

Monitoring of bone marrow and in particular blood postsurgery (i.e., during and after systemic adjuvant therapy) might provide a unique information for the clinical management of the individual cancer patient (62, 63, 78, 79, 88). The identification of patients at increased risk for recurrence after completion of adjuvant chemotherapy is an application of high clinical relevance, because these patients might benefit from an additional “second-line” treatment, e.g., bisphosphonates or targeted therapies like anti-*HER2* approaches or anti-angiogenic drugs.

### Critical Issues of CTC/DTC Detection

**Definition of a positive finding.** Depending on the detection technique used, various internal assay controls (e.g., cell morphology, PCR controls, or EPISPOT morphology) need to be implemented and the diagnostic threshold is usually determined by comparing the findings in a noncancer control group to those found in cancer patients. Interlaboratory

comparisons are then required to test the robustness of the established techniques and the defined cutoff values. There are ongoing activities on the international standardization of immunocytochemical assays (9).<sup>3</sup> Whereas for the immunocytochemical analysis of bone marrow from primary breast cancer patients, already one stained cell—usually verified by morphologic criteria—is considered as a positive finding, the reported cutoff value for the analysis of blood from patients with metastatic breast cancer using the CellSearch system is higher (23, 26). However, this value seems to depend on the stringency of criteria used for the assay readout (24).

As Butler and Gullino already stated in 1975, entry of tumor cells into the bloodstream is a frequent event (89); however, even cells that have been selected for metastatic capacity very rarely develop metastases (90, 91). Therefore, only the comparison of the established assay findings with the clinical outcome will define the clinically relevant threshold. Large-scale immunocytochemical bone marrow analyses have shown that the detection of only one stained cell per sample is already prognostically relevant in patients with primary breast cancer if appropriate antibodies are applied (10), whereas the prognostically relevant cutoff value for blood analyses by the CellSearch system in patients with metastatic breast cancer was reported to be five cells (23).

**Reproducibility of the findings.** The information on the reproducibility of CTC/DTC detection assays is very limited. This is at least in part due to the fact that the reproducibility of rare event assays is hampered by the Poisson distribution of these events (i.e., stochastic chance to miss an event), which can easily lead to false-negative findings in repeat analysis. Assay reproducibility experiments are usually done by the analysis of bone marrow or blood samples added to a defined number of tumor cell line cells (25). Although these experiments are important, they provide only limited information about the reproducibility of taking samples from cancer patients, which may depend on the biology of CTC/DTC *in situ* and additional variables such as the sample volume and the disease stage of the patients. Whereas the CTC analysis of 7.5 mL blood seems to provide reproducible results in metastatic cancer patients (25, 92), larger volumes are needed in patients without overt metastases where the total burden of CTC (and DTC) is obviously lower.

**Negative findings in metastatic patients.** A surprising observation is the considerable percentage of negative findings of CTC analyses in the peripheral blood of patients with overt metastases. The rate of these findings depends on the detection technique used and ranges from 10% to 50% (23–25, 37, 80, 93). CTC might be missed because (a) the detection marker is not expressed in these cells, maybe due to dedifferentiation and/or a process called epithelial-to-mesenchymal transition (94); (b) the frequency of CTC is below the threshold of detection; and (c) the release rate of CTC from overt metastases might be variable, which could be an interesting subject of future investigations with potential implications for new forms of therapies blocking secondary dissemination of metastatic cells.

**Escape from detection by phenotypic changes of tumor cells.** The interpretation of CTC/DTC assays may be also misled by intrinsic changes in the phenotype of disseminating tumor cells. As an example, loss of expression of epithelial marker proteins used for CTC/DTC detection (e.g., epithelial cell adhesion molecule or cytokeratin) may occur during

epithelial-to-mesenchymal transition. Moreover, changes induced by the applied therapy may lead to false-negative findings, which might be misinterpreted as elimination of CTC/DTC. This issue is most relevant for the increasing use of real-time CTC monitoring to assess therapeutic efficacy. Taking these notes of caution into account, multimarker approaches or the use of single antibodies that recognizes common epitopes present on various cytokeratin proteins should be superior over single marker assays.

**Unknown biology of tumor cell shedding.** Release of cancer cells from the primary tumor has been called "shedding." Epithelial cells are usually tightly fixed in their tissue of origin by strong adhesion junctions, but decreased intercellular adhesion in tumor tissues might account for increased cell shedding at the tumor surface. Recent gene expression profiling studies suggested, however, that early dissemination of breast cancer cells into the bone marrow and the release of these cells into the circulation might be a rather active and selective process associated with a specific signature of the primary tumor (50, 51).

Another important open question is whether the tumor cell release is a continuous or sporadic process (maybe even with a circadian rhythm). The daily rate of shedding in cancer patients is also unknown but estimates of rather high rates have been made from experimental studies. Thus, MTW9 rat mammary carcinomas shed  $3 \times 10^6$  to  $4 \times 10^6$  tumor cells per 24 hours per gram of tissue into the tumor efferent blood (89). It is questionable that these data are directly applicable to cancer patients because, in contrast to animal models, most of their tumors grow slowly and these tumors would probably disappear if a high loss of cells into the circulation would take place every day (95). Estimates on the half-life time of CTC in the range of 1 to 2.4 hours have been made by Jonathan Uhr's group (96).

Another debated topic is whether tumor cell release can be induced or augmented by medical treatments. Induction of tumor cell shedding by surgical manipulation has already been reported many years ago (97–99) but there are only limited data on the clinical relevance (100). Tumor cells that are not biologically selected for dissemination but only mechanically released may more easily undergo anoikis upon loss of their adhesive contacts (101, 102) or lack relevant homing receptors such as CXCR4. It was also shown that tumor cells were comobilized with hematopoietic stem cells by treatment with granulocyte colony stimulating factor and low-dose chemotherapy (103), and many efforts were undertaken to "purge" the stem cell transplants from these CTC.

**Can bone marrow analyses be replaced by CTC blood measurements?** Sequential peripheral blood analyses should be more acceptable than bone marrow aspirations and many research groups are currently assessing CTC in clinical studies. In metastatic breast cancer patients, a repeated sampling of bone marrow is almost obsolete, and the detection of CTC has provided significant prognostic information (23, 104) and

seems to be superior over conventional imaging methods for response evaluation (27). In contrast, the prognostic relevance of CTC in the blood of patients with early-stage disease without overt metastasis is still under investigation, with encouraging results from smaller single-center studies (105–108).

It is still not clear if CTC measurements could replace the examination of the bone marrow. Previously, two immunocytochemical studies showed statistically significant correlations between DTC detection in bone marrow and blood but bone marrow was more frequently positive than blood (80, 109). One possible explanation is that bone marrow is a homing organ for DTC, whereas blood analyses allow only a "snapshot" of tumor cell dissemination. Recently, it was also described that detection of DTC in bone marrow had superior prognostic significance in comparison with CTC measurements in blood, analyzing patients with metastatic and nonmetastatic breast cancer by a quantitative RT-PCR assay for CK19 and mamoglobin mRNAs (40). Consistently, another report using immunocytochemistry showed that only bone marrow but not blood analyses provided prognostic information (110). Currently, these findings do not support an exchange of DTC in bone marrow with CTC from blood in breast cancer but future studies with improved detection technologies may help to clarify this issue. In other tumor entities such as colon or pancreatic cancer where overt bone marrow metastases are rare, CTC analysis have also revealed prognostic information (111, 112).

## Conclusions

Detection and characterization of CTC/DTC can be used for estimation of the risk for metastatic relapse (prognostic information), stratification of patients to adjuvant therapy, identification of therapeutic targets, and monitoring of systemic anticancer therapies. Moreover, the detection and molecular characterization of early disseminated cancer cells provide new insights into the biology of metastatic development in cancer patients. These data can then be cross-validated with the findings in experimental models, which allow sophisticated functional analyses. A better understanding of the complex metastatic process may lead to the discovery of new targets for antimetastasis therapies.

In clinical oncology, there is an urgent need for biomarkers for real-time monitoring of the efficacy of systemic adjuvant therapy in individual patients, such as the blood glucose test for directing insulin treatment of diabetes. Research on CTC/DTC has the potential to fill this important gap in oncology.

## Disclosure of Potential Conflicts of Interest

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