

Biologic Challenges in the Detection of Circulating Tumor Cells

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Abstract

Carcinoma cells found in the blood of cancer patients are predictors of metastatic progression and may guide treatment decisions. Most of the current strategies for detecting circulating tumor cells (CTC) are based on the epithelial markers epithelial cell adhesion molecule and keratin; however, evidence is accumulating that in certain tumor types, these epithelial markers are downregulated during tumor cell dissemination, hampering the detection of CTCs. This short review discusses the implications of the cellular changes of tumor cells during the metastatic cascade on CTC diagnostics. *Cancer Res*; 73(1); 8–11. ©2012 AACR.

Background

Cancer-related death is in general the consequence of tumor cells spreading from the primary tumor and forming metastases in resident organs (1). In routine cancer diagnostics by histopathology and high-resolution imaging technology, it remains unclear whether early tumor spread has taken place until the manifestation of overt metastasis. Therefore, administration for systemic adjuvant therapy for cancer metastasis intervention is presently based on personal statistical risk, resulting in overtreatment of many patients. A novel method to estimate the risk for metastatic relapse or progression is the detection of circulating tumor cells (CTC) in cancer patients' blood by immunocytochemical or molecular assays (2). These assays might also be used to monitor the effectiveness of administered systemic therapy in real-time (3); however, it has come to attention that some patients, although negative for CTCs, still develop metastasis. Moreover, a significant fraction of patients with overt metastasis (in particular, in the brain) have surprisingly low CTC counts. One of the explanations is that some CTCs that are capable of forming metastasis escape the current detection methods and remain undetected. In this article, we will review the presently used methods and challenges for identifying CTCs and discuss the future perspectives in relation to cancer biology.

Current principles of CTC detection

To detect the few rare malignant cells in several milliliters of blood containing billions of red blood cells and tens of millions of leukocytes, extremely sensitive and specific methods are required that are able to process the large amount of cells in a

relatively short period of time. Most detection assays are preceded by enrichment steps that generally involve removal of red blood cells by density-gradient centrifugation or erythrocyte lysis, followed by immunomagnetic bead or immunofluorescence-based separation of mononuclear cells. Parting the tumor cells from the leukocytes is usually conducted by positive enrichment for epithelial cells using the commonly used surface marker epithelial cell adhesion molecule (EpCAM), or by negative depletion of hematopoietic CD45 positive cells. Other techniques for CTC enrichment or isolation make use of size, density, electric charges, or deformability and have been outlined elsewhere (4). A final verification for CTCs is usually conducted by the identification of protein or mRNA expression of keratins (formerly known as cytokeratins). Hence, many of the currently used techniques to detect CTCs, including the U.S. Food and Drug Administration cleared CellSearch System, make use of the epithelial cell markers EpCAM and keratins, combined with the criteria of CD45 negativity and having a cell nucleus. Lately, however, these criteria have become a point of discussion: although the prognostic value of EpCAM/keratin positive CTCs has been proven, it is nevertheless the question whether more cells with metastatic potential can be detected by other or a broader range of markers.

Biology of tumor cell dissemination

Not all invasive tumors will lead to the formation of metastasis (5). The understanding on how and which tumors might give rise to metastasis has increased enormously in the last decade but still lacks much specificity. The first step that marks the metastatic cascade is the invasion of surrounding tissue by individual or small clusters of tumor cells (6). To migrate, carcinoma cells have to lose several of their epithelial characteristics that bind them to surrounding cells and the basement membrane. This requires for changes in cell adhesion, activation of proteolysis, and acquiring motile properties. After local invasion, the next step of the metastatic cascade is entrance of the lymphatic system or blood circulation (intravasation). A large number of cells enter the bloodstream of cancer patients continuously, but most cells are dead, apoptotic, die as a consequence of shearing

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force or anoikis, or are eliminated by the immune system. The small proportion of CTCs that survives and are capable of leaving the bloodstream can either extravasate foreign tissue or reenter the primary tumor site (self-seeding; ref. 7). Finally, the disseminated tumor cells grow out to form a metastasis, die, or remain dormant for many years.

The consequence of EMT for CTC detection

The dedifferentiation program epithelial–mesenchymal transition (EMT) is thought to be responsible for the first step in the metastatic cascade and allows cells to become motile (8, 9). During this process, epithelial markers such as cell adhesion molecules and tight junctions (e.g., E-cadherin) are downregulated to lose cell–cell contact. EpCAM is expressed in healthy individuals on most epithelial cells, making it the best known epithelial cell surface marker. The protein is able to mediate homophilic adhesive interactions and would, therefore, prevent cell scatter. Concordantly, it has been shown that downregulation of EpCAM is required for breast cancer invasion, which takes place during EMT (10). However, contradictory to this are EpCAM expressing pancreatic cancer cells that have an increased tumorigenic potential compared with EpCAM negative pancreatic cancer cells (11). In other cancer types, the expression of EpCAM can be related to oncogenic, tumor suppressing, or even both phenotypes, suggesting that the role of EpCAM in tumor promotion is dependent on tumor type and/or the metastatic environment and a more sophisticated enrichment method for CTCs is required and possibly independent from EpCAM.

Another noticeable feature of EMT is the reorganization of the cytoskeleton by changes in intermediate filaments to improve cell motility. Keratins are a large family of intermediate filaments and are primarily present in epithelial cells. Intermediate filaments normally extend through the cytoplasm to provide tensile strength to cells and collaborate to cell–cell adhesion by interaction with desmosomes. The expression of specific keratins is tissue- and differentiation state-dependent; most adenocarcinomas and epithelial cancers originating from glandular tissue express K8, K18, and K19. Therefore, keratins are frequently used for histotyping carcinomas to aid the selection of the most appropriate treatment plan, and currently, to specifically identify CTCs/disseminated tumor cells (DTC). A point of discussion in using keratins for detecting CTCs is that several of the keratins are often downregulated in high-grade breast carcinomas. Moreover, it is speculated that keratin expression changes during EMT (10, 12). In a recent study, we have seen that keratin expression in lymph node metastasis is frequently altered compared with the corresponding primary breast carcinoma. However, it was noticeable that not all keratins were downregulated and with a cocktail of antibodies against a broad range of keratins, carcinoma cells can still easily be detected as well as a higher number of CTCs in metastatic breast cancer patients as compared with the conventional used keratin antibodies (12).

From CTC detection to MIC identification

Although some prognostic information can already be obtained by the detection of EpCAM/keratin-positive CTCs

alone, further improvement can be made in the field of CTC diagnostics to determine the precise metastatic risk in individual cancer patients. First of all, it is debated whether the EpCAM/keratin-positive CTCs may or may not have any metastatic potential because of their lack of apparent plasticity. It is thought that epithelial cells undergo EMT to become motile and invade the surrounding tissue (8). Therefore, it might be speculated that tumor cells that apparently did not undergo EMT, and still express EpCAM and keratin immutably, have detached from the primary tumor and entered the bloodstream via a passive process. Several models have been postulated explaining the migration of tumor cells through the surrounding tissue (13). It is plausible that tumor cells, after EMT, generate a proteolytic microtrack to migrate along which trailing epithelial cancer cells can follow (Fig. 1). Consequently, both (semi)-mesenchymal and epithelial cancer cells enter the blood stream. It has been shown that the cells with an increased epithelial–mesenchymal plasticity and mobility are cancer stem cells (CSC) that initiate the growth of the primary tumor (14). It is, therefore, likely that the so-called metastasis-initiating cells (MIC) arise from circulating CSCs with an EMT phenotype (15). Thus, the current assays focused on epithelial markers downregulated during EMT (e.g., EpCAM and keratins) might be only surrogate markers of the presence of MICs but may not detect MICs themselves. This would explain why the current assays based on epithelial markers can be used as biomarkers but nevertheless, they might fail to provide meaningful information on those tumor cells (MICs) that are the most relevant targets of therapeutic interventions. Future studies using CTC assays that detect (semi)-mesenchymal CTCs that underwent an EMT will test this provocative hypothesis in the context of targeted therapies.

The next important aspect of epithelial–mesenchymal plasticity is the switch from EMT to MET, leading to the final steps to colonization of distant organs. Recent studies indicated that EMT can suppress major attributes of human epithelial tumor-initiating cells and concluded that the dynamic interactions among epithelial, self-renewal, and mesenchymal gene programs determine the plasticity of epithelial tumor cells (16). Thus, CTCs "frozen" in an epithelial or mesenchymal state might be less "dangerous" than tumor cells with a high plasticity that are able to switch between EMT/MET depending on certain conditions such as (re-)expression of E-cadherin or EMT-inhibitory miR-200s that are important for the initiation of metastatic relapse (17–20). Future CTC assays might, therefore, need to identify tumor cells with the highest degree of plasticity. Current methods to detect MICs that are under investigation make use of stem cell markers such as CD44, CD24, CD133, and ALDH-1; still, not even stem cell-like CTCs might survive the systemic circulation that is essential for homing to distant organs. For such reasons a technique called EPISPOT has been developed that is able to detect viable cells without selecting for EpCAM-positive cells but using excreted proteins such as keratin 19, MUC1, and prostate-specific antigen (PSA) as tumor marker for CTC detection. Using this technology, the release of the stem cell growth factor FGF-2 of PSA-secreting CTCs from prostate cancer

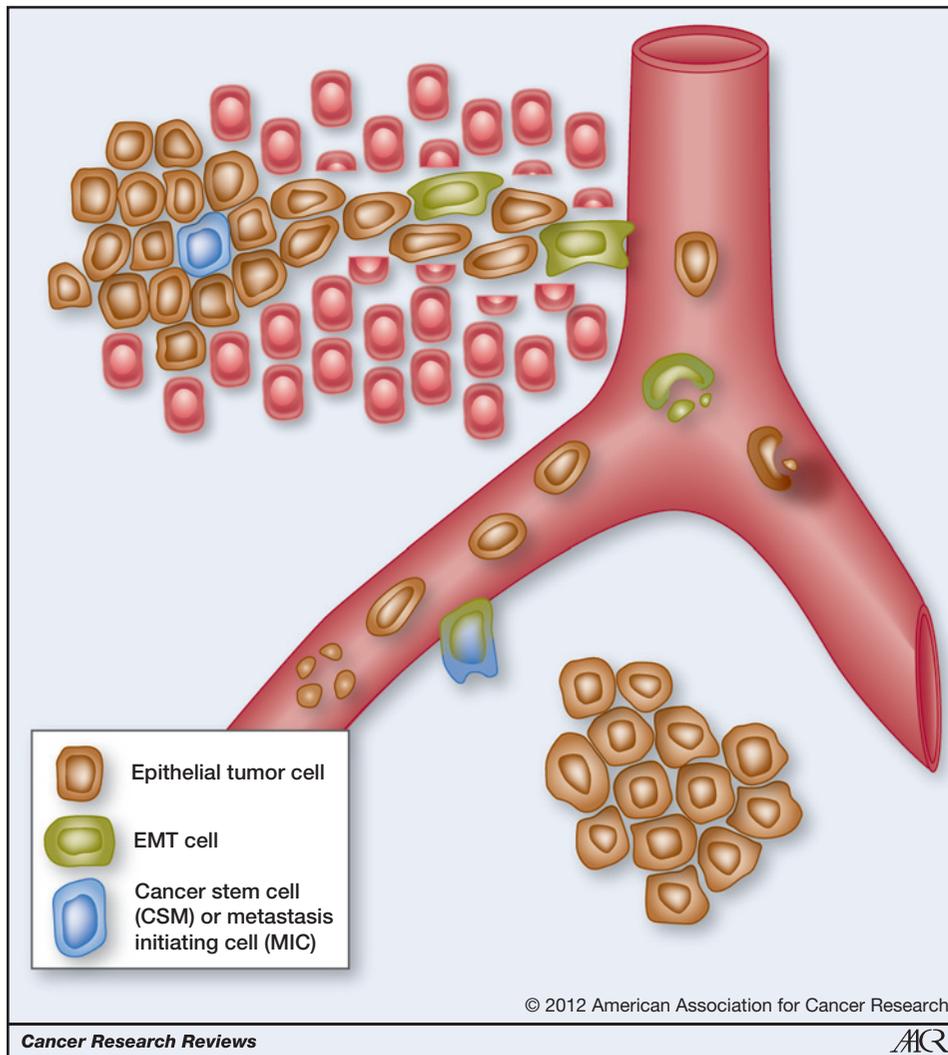


Figure 1. Model of metastasis initiating cells. The growth of the primary tumor (upper left) is driven by CSCs, which have a high plasticity and may undergo EMT. CSCs that underwent EMT infiltrate the surrounding tissue, forming a proteolytic microtrack in which trailing epithelial cancer cells follow. As a consequence, CSCs and non-CSCs cells enter the blood circulation, in which many tumor cells die. CSCs may have an increased ability to survive and extravasate the blood stream, and in their new resident organ, they may become MICs. Interestingly, to form an overt metastasis, MICs need to switch back to an epithelial phenotype (bottom). If this model is correct, CTC detection methods relying only on epithelial markers would miss circulating MICs.

patients could be detected (21). In the future, it will be important to develop more functional assays to decipher the biology of CTCs.

Another issue of discussion regards the time point at which tumor cells leave the primary site and infiltrate the body. This is clinically very important as MICs released at early stage might develop independently from the primary tumor exhibiting different properties such as therapy responses. On the other hand, late-stage dissemination might implicate the chance of systemic overtreatment prevention of cancer patients that underwent primary surgery at an early stage of tumor development when tumor cells have not invaded the body yet. The discussion about early or late stage dissemination is based on 2 main models that explain the progression of primary cancer to the metastatic stage by tumor cell dissemination (22): the linear progression model describes a stepwise progression of multiple rounds of genetic mutations, epigenetic changes, and selection for competitive fitness. After a significant number of such rounds, cells might be able to infiltrate, to proliferate autonomously, and to form metastases in distant organs. On

the other hand, the parallel progression model concludes that tumor cells are released at an early state of cancer progression and that primary tumor and metastasis grow in parallel, long before the first symptoms of cancer appear. In favor of the later model is the growth rate of cancer leading to the size of metastases that remain unexplained with late stage dissemination. Early dissemination has been confirmed in recent studies in which viable CTCs could be detected in patients with DCIS (23) and benign colon diseases (24), and in a mouse model for pancreatic cancer even before an overt malignant tumor became detectable (25). Nevertheless, CTCs detected at early stage might not have metastatic potential yet and indeed studies conducted on pancreatic tumors showed that MIC clones developed only at late stage (15, 26). Furthermore, most DTCs detected in bone marrow of cancer patients are Ki67-negative (i.e., nonproliferating; ref. 1) and proliferation is required to acquire mutations and pass them on to the daughter cells. Nevertheless, a snapshot analysis of DTCs may not exclude that proliferation of these cells occurs at a low rate and metastasis formation in patients can take many years.

Conclusion

The future perspectives in CTC detection lie in the identification of markers specifying the metastatic potential of single CTCs and the selection and in-depth analysis of MICs with emphasis on druggable targets. Upon success, a blood sample might be used as a liquid biopsy in tumor diagnostics that is easily accessible for both patient and physician.

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Authors' Contributions

Conception and design: S.A. Joosse, K. Pantel

Development of methodology: K. Pantel

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.A. Joosse, K. Pantel

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.A. Joosse, K. Pantel

Writing, review, and/or revision of the manuscript: S.A. Joosse, K. Pantel

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.A. Joosse, K. Pantel

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References

- Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. *Nat Rev Cancer* 2004;4:448–56.
- Miller MC, Doyle GV, Terstappen LW. Significance of circulating tumor cells detected by the CellSearch system in patients with metastatic breast colorectal and prostate cancer. *J Oncol* 2010;2010:617421.
- Lianidou ES, Markou A. Circulating tumor cells in breast cancer: detection systems, molecular characterization, and future challenges. *Clin Chem* 2011;57:1242–55.
- Bednarz-Knoll N, Alix-Panabieres C, Pantel K. Clinical relevance and biology of circulating tumor cells. *Breast Cancer Res* 2011;13:228.
- Wicha MS, Hayes DF. Circulating tumor cells: not all detected cells are bad and not all bad cells are detected. *J Clin Oncol* 2011;29:1508–11.
- Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer* 2002;2:563–72.
- Kim MY, Oskarsson T, Acharyya S, Nguyen DX, Zhang XH, Norton L, et al. Tumor self-seeding by circulating cancer cells. *Cell* 2009;139:1315–26.
- Bonnomet A, Brysse A, Tachsidis A, Waltham M, Thompson EW, Polette M, et al. Epithelial-to-mesenchymal transitions and circulating tumor cells. *J Mammary Gland Biol Neoplasia* 2010;15:261–73.
- Gunasinghe NP, Wells A, Thompson EW, Hugo HJ. Mesenchymal-epithelial transition (MET) as a mechanism for metastatic colonisation in breast cancer. *Cancer Metastasis Rev* 2012;31:469–78.
- Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. *Proc Natl Acad Sci U S A* 2010;107:15449–54.
- van der Gun BT, Melchers LJ, Ruiters MH, de Leij LF, McLaughlin PM, Rots MG. EpCAM in carcinogenesis: the good, the bad or the ugly. *Carcinogenesis* 2010;31:1913–21.
- Joosse SA, Hannemann J, Spotter J, Bauche A, Andreas A, Muller V, et al. Changes in keratin expression during metastatic progression of breast cancer: impact on the detection of circulating tumor cells. *Clin Cancer Res* 2012;18:993–1003.
- Friedl P, Alexander S. Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell* 2011;147:992–1009.
- Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008;133:704–15.
- Baccelli I, Trumpp A. The evolving concept of cancer and metastasis stem cells. *J Cell Biol* 2012;198:281–93.
- Celia-Terrassa T, Meca-Cortes O, Mateo F, de Paz AM, Rubio N, Arnal-Estape A, et al. Epithelial-mesenchymal transition can suppress major attributes of human epithelial tumor-initiating cells. *J Clin Invest* 2012;122:1849–68.
- Chao YL, Shepard CR, Wells A. Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition. *Mol Cancer* 2010;9:179.
- Korpala M, Eli BJ, Buffa FM, Ibrahim T, Blanco MA, Celia-Terrassa T, et al. Direct targeting of Sec23a by miR-200s influences cancer cell secretome and promotes metastatic colonization. *Nat Med* 2011;17:1101–8.
- Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* 2010;467:1114–7.
- Park D, Karesen R, Axcrone U, Noren T, Sauer T. Expression pattern of adhesion molecules (E-cadherin, alpha-, beta-, gamma-catenin and claudin-7), their influence on survival in primary breast carcinoma, and their corresponding axillary lymph node metastasis. *APMIS*. 2007;115:52–65.
- Alix-Panabieres C. EPISPOT assay: detection of viable DTCs/CTCs in solid tumor patients. *Recent Results Cancer Res* 2012;195:69–76.
- Klein CA. Parallel progression of primary tumours and metastases. *Nat Rev Cancer*. 2009;9:302–12.
- Sanger N, Effenberger KE, Riethdorf S, Van Haasteren V, Gauwerky J, Wiegatz I, et al. Disseminated tumor cells in the bone marrow of patients with ductal carcinoma in situ. *Int J Cancer* 2011;129:2522–6.
- Pantel K, Deneve E, Nocca D, Coffy A, Vendrell JP, Maudelonde T, et al. Circulating epithelial cells in patients with benign colon diseases. *Clin Chem*. 2012;58:936–40.
- Rhim AD, Mirek ET, Aiello NM, Maitra A, Bailey JM, McAllister F, et al. EMT and dissemination precede pancreatic tumor formation. *Cell* 2012;148:349–61.
- Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* 2010;467:1114–7.

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