Detection of circulating tumor cells: Clinical relevance of a novel metastatic tumor marker (Review)

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Abstract. Most cancer-related deaths are caused by the hematogenous spread of cancer cells to distant organs and their subsequent metastasis. During the early stages of the metastatic cascade, cancer cells disseminate from the primary site via the lymphatic vessels and/or by hematogenous routes. Circulating tumor cells (CTCs), cancer cells that have disseminated into the systemic circulation, may be a predictor of poor prognosis in several carcinomas. An understanding of the molecular mechanisms involved in the blood-borne dissemination of cancer cells may help to clarify the process of metastasis and provide a powerful and non-invasive approach for anticancer treatments that are tailored to individual patients.

Contents

1. Introduction

Metastasis to distant sites (e.g., lungs, liver, bone and brain) via the bloodstream or lymph nodes is a major cause of

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cancer-related mortality (1-3). Circulating tumor cells (CTCs) play an important role in cancer relapse and metastasis. CTCs identical to those in primary tumors were first discovered by Ashworth as early as 1869 (4), and were later regarded as a hallmark of the 'leukemic phase' of cancer (5). CTCs were proposed as a novel minimally invasive prognostic and predictive marker that reflects the biological characteristics of tumors, and have been the subject of an increasing number of clinical studies. Identifying cancer cells among the millions of normal blood cells during the early stages of cancer, however, is challenging. In recent years, many new methods have been developed to enrich and detect these rare CTCs in peripheral blood (6-13). The different technologies involved, coupled with the heterogeneity of the screened populations, make the clinical significance of CTCs difficult to interpret (7). Thus, it is necessary to standardize the detection methods used to identify CTCs in order to determine their biological and clinical relevance. The detection of dynamic changes and malignant features within these rare cells is closely associated with the efficacy of therapy and with prognosis (6,14-24). CTCs may play an important role in the detection of early relapse and in the assessment of prognosis and the efficacy of the chosen therapy for both established cancers and metastatic precursor cells.

2. Malignant features of metastatic CTCs

Occult tumor cells may persist in a dormant or low prolif-erative state after curative therapy. It is these cells that are responsible for tumor relapse and metastasis. Such cells, which are not detectable by current routine diagnostic methods, may play an important role in recurrence as they may express different biological characteristics and/or markers from those of the primary tumor (25). Therefore, the detection and characterization of CTCs is of the utmost clinical relevance. The processes by which cancer cells proliferate [angiogenesis, detachment from the primary tumor, epithelial to mesenchymal transition (EMT) and intravasation into the vasculature followed by extravasation into distal organs] are not yet fully understood (Fig. 1). As cancer cells invade through the basement membrane, they undergo EMT and are shed into the circulation. This process is vital for metastasis.

Table I. Detection of CTCs in solid tumors and prognosis.

Tumor type	TNM	Samples (n)	Methods	Markers	Positivity of CTC (%)	Prognosis	Refs.
Breast cancer	IV	80	CellSearch	Ck8/18/19+ CD45- cells	61.0	PFS	16
Breast cancer	IV	177	CellSearch	CK8/18/19+ CD45- cells	49.0	PFS OS	19
Breast cancer	I-II	148	RT-PCR	CK19 mRNA	29.7	DFS OS	66
Breast cancer	I-II	444	RT-PCR	CK19 mRNA	40.8	DFS OS	67
Hepatic cancer	I-IV	101	RT-PCR	Albumin mRNA	45.0	DFS no OS no	78
Colorectal cancer	I-IV	196	RT-PCR	CEA, CK19 mRNA	85.0	PFS	68
Colorectal cancer	I-II	66	RT-PCR	CEA mRNA	54.5	OS no	79
Colorectal cancer	IV	413	CellSearch	CK8/18/19+ CD45- cells	26 ≥3 CTCs	OS PFS yes	51,69
Prostate cancer	IV	162	RT-PCR	PSA mRNA	44.0	OS	70
Bladder cancer	T1G3	54	RT-PCR	Survivin mRNA	44.0	PFS	76
Non-small cell lung cancer	I-II	61	RT-PCR	TIF-1 CK19 mRNA	36.1 42.6	PFS	77

PFS, progression-free survival; OS, overall survival; DFS, disease-free survival.

Due to changes in the microenvironment and destruction by shearing forces in the blood vessels and by immune cells, most CTCs in the circulation undergo apoptosis. The result is that only a very small proportion of CTCs survive (5). However, given ideal conditions, they may extravasate and develop into micrometastases (26). A mouse model of tumor metastasis showed that 10^6 cancer cells were shed into the blood stream every day, and that most of these failed to form metastases due to cellular apoptosis (27).

The malignant phenotype of CTCs is closely related to their metastatic tendency. CTCs derived from breast cancer, renal cell carcinoma, prostate cancer, colon cancer and melanoma have been shown to be malignant by analyzing the aneusomy of chromosomes 1, 3, 4, 7, 8, 11 or 17 using dual or tricolor fluorescence *in situ* hybridization (FISH) (28,29). During the early stages of tumor formation, hypoxia triggers neovascularization within the lesions, which facilitates tumor dissemination via the blood vessels. This process occurs prior to proliferation and, of course, before the appearance of clinical symptoms in the patients (30). Therefore, early detection of CTCs may enable the early diagnosis of cancer. As CTCs are shed into the blood of breast cancer patients every few hours, apoptotic CTCs are replenished by cells originating from the primary tumor, thus maintaining a balance between apoptotic and proliferating CTCs. Several CTCs may remain in the circulation for up to 22 years, which may explain tumor dormancy (31).

Studies of the signaling pathways within CTCs have found that breast cancer CTCs co-express p-FAK, p-PI3K and HER2, suggesting that they possess activated protein kinases, which

regulate their metastatic ability (32,33). Analysis of these proliferative or metastatic signaling pathways may help to elucidate the mechanisms underlying the malignant biology of CTCs.

Whether CTCs metastasize to other organs depends on their genetic profile. The metastatic potential of CTCs is closely related to their heterogeneity, the microenvironment and the efficiency of the patient's immune system. The immune system sees CTCs as 'foreign', but many CTCs escape immune surveillance and manage to form micrometastases and macrometastases in distant organs (34).

3. Methods for separating CTCs

Recent technological advances in the detection and characterization of CTCs have proven helpful in understanding the biology and clinical significance of these rare cells. Many methods can be used to enrich and identify CTCs (35), such as immunomagnetic cell enrichment (which includes magnetic activated cell separation; MACS) (36), the CellSearch system, isolation by the size of epithelial tumor cells (ISET) (37,38), epithelial immunospot (EPISPOT), immunological assays based on enzyme-linked immunosorbent assay (ELISPOT) technology (7) and microchips that enrich CTCs from millions of white blood cells (12,13) (Table I).

4. CTC enrichment

CTCs in the peripheral blood of patients with solid tumors are rare, and so the sensitivity and specificity of detection is dependent upon the particular technological approach used.
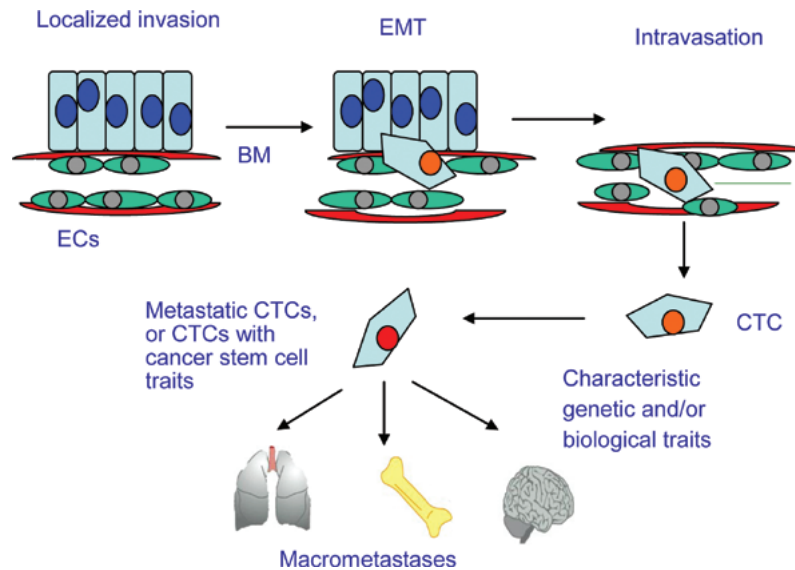
Figure 1. The process of CTC metastasis. Cancer cells with localized invasion require epithelial mesenchymal transition (EMT), by which adherent cancer cells achieve the ability to migrate, as well as with the loss of integrity of the basement membrane (BM) and the extracellular matrix (ECM). The process permits some metastatic cancer cells to intravasate into the blood. CTCs interact with the microenvironment in the circulation. Finally, only a small proportion of CTCs with the ability to metastasize, or those with traits similar to those of cancer stem cells, are thought to extravasate to distal organs and develop macrometastases. Genetic and molecular analysis of these rare cells may provide a novel tool for evaluating their biological and clinical relevance.
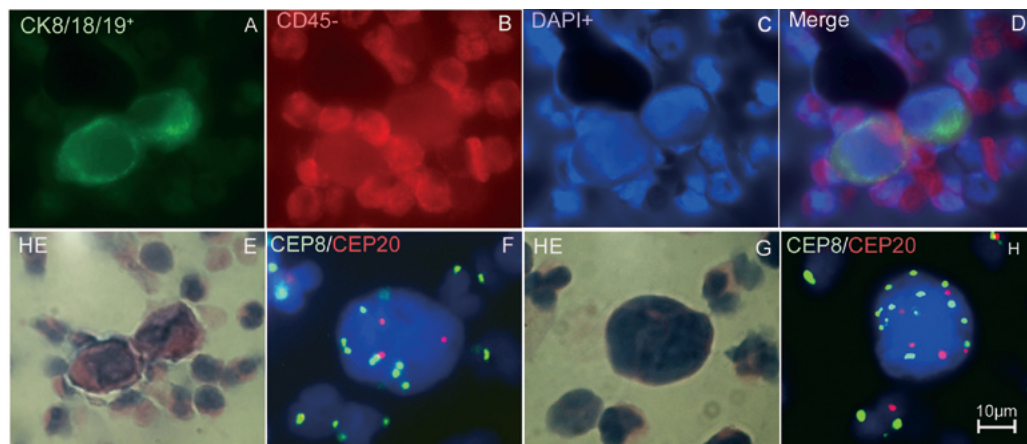

Figure 2. Enrichment and identification of CTCs. CTCs were stained with (A) anti-cytokeratin 8/18/19-Alexa 488-positive, (B) anti-CD45-Alexa 594-negative, (C) 4,6-diamidino-2-phenylindole (DAPI)-positive antibodies. (D) Merged image of A, B and C; (E and G) CTCs re-stained with Hematoxylin and Eosin (H&E). (F and H) CTCs hybridized with DNA probes targeting specific chromosome 8 (green) and chromosome 20 (red), showing polysomy of chromosomes 8 and 20. Original magnification, x100. Scale bar, 10 μm.

In the past, immunomagnetic methods of enrichment have been widely used (9,11). Basically, these techniques involve either positive or negative selection of the chosen cell type. For positive selection, beads are linked to the epithelial antibody, EpCAM, to enrich the rare cells. For negative selection, beads are linked the common leukocyte antigen, CD45, in order to deplete the hematopoietic cells (11). Positive enrichment systems include the CellSearch system (approved by the FDA in 2004), CTC-chips, MACS (when used for positive selection) and the OncoQuick system. The major negative enrichment system is MACS. Although many methods enable the isolation of CTCs with an epithelial phenotype, such as EpCAM and CK antigen expression, the disadvantage is that epithelial proteins are down-regulated during EMT (39), which may affect the efficiency of CTC detection (Fig. 2). Therefore, positive enrich-

ment strategies may not detect EpCAM-negative CTCs (40). A comparison of two different methods for enumerating CTCs in carcinoma patients showed that the CellSearch system (mean detection rate, 20 CTCs/7.5 ml of blood) is a more accurate and sensitive method for enumerating CTCs than the OncoQuick system (mean detection rate 3, CTCs/7.5 ml; P<0.0001) (41,42).

The newly developed microchip technology provides the highest detection rate for CTCs. The chip separates CTCs from whole blood using EpCAM-coated micro-posts under controlled laminar flow conditions to ensure optimal interaction between the cells (12,35,43). The purity of the CTCs was found to be >100 times that obtained using other methods (12). However, this enrichment system requires further clinical validation of its accuracy. In cancer cell spiking experiments using the CellSearch system, recovery rates were 85 and 92%, respec-

Table II. Methods for CTC analysis.

System	Blood volume per test (ml)	Principle of enrichment	Principle of identification	Sensitivity	Recovery rate (%)	Refs.
OncoQuick	10-15	Density centrifugation and a porous barrier membrane	Cytometry or RT-PCR	1 CTC/9.5×10^4 WBC	70-90	41,42
ISET	10	Cell size	CK19 RT-PCR	1 CTC/2×10^6 WBC	NS	37,38
MACS	5-16	Depletion of leukocytes or enrichment of epithelial originated cells using immunobeads	CK8/18$^+$ cells	1 CTC/1×10^6 WBC	NS	36
CellSearch (Veridex)	7.5	Beads coated with EpCAM	CK8/CK18/CK19$^+$ and CD45$^-$ cells	1 CTC/1×10^7 WBC	85	45
CTC microchip	5	Microposts coated with EpCAM	CK8/CK18/CK19$^+$ and CD45$^-$ cells	1 CTC/1×10^7 WBC	>65	43
FAST		Beads coated with EpCAM	CK$^+$ cells	10^{-6}	>86	57

NS, non-significant (data not shown). WBC, white blood cells.

tively, from two different patient groups (44,45). New CTC-chip micro-fluidic technology showed a 10- to 100-fold improvement in CTC yield from patient samples over the CellSearch system (44). However, this method needs further validation using large numbers of clinical samples. Since not all CTCs express EpCAM, the use of magnetic beads labeled with both CD146 and EpCAM antibodies increases the rate of detection (46).

5. CTC identification

The key to successfully identifying CTCs is differentiating them from other hematopoietic cells and squamous cells. CTCs can be identified as malignant by cytomorphology, tumor-specific antigen expression and aneusomy of the chromosomes (47). The techniques used to identify CTCs are broadly divided into cytometric- and nucleic acid-based approaches (Table II). Cytometric approaches use immuno-cytochemical methods to characterize individual tumor cells. Nucleic acid-based approaches detect DNA or RNA sequences that are differentially expressed in tumor cells and normal controls (11). However, pseudogenes or non-specific sequences may be identified using nucleic acid-based approaches. When the efficacy of the therapy or the tumor burden is to be evaluated, nucleic acid-based approaches may be a simple and straightforward choice. If intact cellular morphology and genetic phenotype are to be studied, cytometric approaches may be preferred (48). FISH has been used to directly identify circulating genetically abnormal cells (CACs) in the peripheral blood of patients with non-small cell lung cancer. Depending on the expression levels of abnormal biomarkers, up to 45 CACs per microliter were detected, compared to <10 CACs per milliliter in most studies using immunomagnetic beads (22,49-53). Peripheral blood-based membrane-array assays with a panel of tumor-related mRNA markers (hTERT, CK-19, CEA and MUC1) were used to identify CTCs in gastric cancer patients using a nucleic acid-based approach

(54). This technique has a satisfactory level of sensitivity and specificity (54). *In vivo*, non-invasive label-free detection and eradication of circulating metastatic melanoma cells using two-color photoacoustic flow cytometry and a diode laser has also been attempted (55). Additionally, GFP-expressing virus-based methods are remarkably simple and allow the precise enumeration of viable CTCs (56). Another approach used is fiber-optic array scanning technology (FAST), which applies laser-printing techniques to the detection of rare CTCs. The combination of FAST enrichment and automated digital microscopy (ADM) imaging yields the level of performance required for the reliable detection of metastatic colorectal cancer cells in the blood (57,58). Further testing using clinical samples and integration of all the modules into a single, fully automated smart miniaturized system will enable minimally invasive testing for the detection and characterization of CTCs (25).

6. CTCs and cancer stem cells

The cancer stem cell theory suggests that only a small fraction of cancer cells are stem cells capable of self-differentiation and self-replication. A few hundred cancer stem cells (CSCs) were found to cause carcinogenesis in NOD/SCID mice, whereas non-cancer stem cells did not (3). The proposed existence of rare CSCs within an ordinary tumor cell population with limited proliferative potential implies that such rare progenitors have the real ability to metastasize (59). CD133, CD44$^+$/CD24$^{-/low}$ and CXCR4 have been proposed as markers for CSCs in glioma, breast, colon, prostate, pancreatic and esophageal cancer (60). CD133 mRNA detected in the peripheral blood of patients with colon cancer is a predictor of poor prognosis (61). CD44$^+$/CD24$^{-/low}$ CTCs have also been identified in the peripheral blood of patients with breast cancer. These putative CSC marker-positive CTCs have been associated with tumor metastasis (62). At present, the relationship between CSCs and

CTCs is not clear. Further research is required to validate any relationship and their clinical relevance.

7. Clinical relevance of CTCs

CTCs may predict tumor relapse, therapeutic efficacy and/or prognosis (Table I). The results of one study found that in case of lung cancer metastasis to distant organs, the number of CTCs clearly increased (63). CTCs may also be surrogate markers for early tumor metastasis (63). In several carcinomas, peripheral blood CTCs were found to be a predictor of poor prognosis (24,64). Cristofanilli *et al* analyzed CTCs in the peripheral blood of 177 metastatic breast cancer patients enrolled in multi-center double-blind prospective studies. The results showed that a detection rate of ≥5 CTCs/7.5 ml peripheral blood indicated a worse prognosis than a rate of <5 CTCs/7.5 ml. CTC dynamics clearly reflected the efficacy of the chosen therapy (19). The basal level of CTCs is a good prognostic indicator, and changes in CTC levels during treatment may reflect the efficacy of the chosen therapy (16). A retrospective study revealed a relationship between the overall survival rate of 37 prostate cancer patients and CTC levels; the overall survival rate of patients with ≥5 CTCs/7.5 ml peripheral blood was 0.7 years compared to 4 years for patients with <5 CTCs/7.5 ml (P=0.002) (65). In patients with breast cancer, CTCs were still detectable in the peripheral blood after the primary tumor was eradicated, and the risk of recurrence in these patients was greater than for those with no detectable CTCs (19). The detection of CK19 mRNA-positive CTCs in the peripheral blood of patients with stage I or II breast cancer prior to adjuvant therapy was an independent prognostic indicator of poor clinical outcome (66), mainly in those patients with ER-negative, triple-negative and HER2-positive early-stage breast cancer (67). CK20 mRNA-positive CTCs detected within 24 h of primary colorectal cancer resection were also found to be a strong predictor of recurrence (68).

The number of CTCs detected by the CellSearch system before and during treatment was found to be an independent predictor of progression-free survival and overall survival in patients with metastatic colorectal cancer. This implies that CTCs may provide prognostic information in addition to the results obtained from imaging studies (51,69). Detection of PSA-positive CTCs is a significant prognostic factor for survival in patients with hormone refractory prostate cancer (70). EGFR expression by CTCs in patients with metastatic breast cancer was also used as a predictive marker for targeted therapy (71). The greatest number of CTCs was detected in patients with esophageal cancer immediately after surgery and correlated with the rate of tumor relapse (72). Wild-type KRAS, detected in CTCs from patients with metastatic colon cancer, strongly correlated with their sensitivity to chemotherapy and with prognosis (73,74). Apoptotic CTCs can be detected in the peripheral blood of patients with prostate cancer after chemotherapy, which may reflect treatment efficacy (75). The presence of surviving CTCs is also an independent prognostic factor in patients with T1G3 bladder cancer (76). Another study demonstrated that TTF-1 mRNA-expressing CTCs may be a useful surrogate predictor of disease progression before clinical symptoms are apparent in non-small cell lung cancer (77).

However, in some solid tumors, the detection of CTCs in the peripheral blood does not predict prognosis. For example, circulating albumin mRNA failed to provide significant information regarding the diagnosis and prognosis of hepatocellular carcinoma (78), and the postoperative detection of blood CTCs using CEA mRNA had no prognostic significance in patients with colorectal cancer after surgical resection (79). The use of CTCs as prognostic indicators in some carcinomas is unreliable. This may be due to the different methods used to detect them and the different populations studied.

8. Advanced tools for tailored therapy?

Dynamic molecular analysis of CTCs may be helpful for targeting therapy in individual patients. HER2 expression is not increased in the primary tumor during the early stages of breast cancer, but increased expression can be detected in CTCs in advanced breast cancer. This may be why patients treated with herceptin have a good prognosis (80). The molecular genetics of CTCs are similar to those of the primary tumor. Therefore, CTCs may represent the status of the primary tumor (81). Nearly 98% of patients with HER2-positive primary and metastatic cancers had CTCs expressing elevated levels of HER2. However, 33% of patients, in whom the primary cancer was HER2-negative, had CTCs that were HER2-positive (44). This suggests that HER2 expression by CTCs may provide the rationale for individually targeted HER2 therapy (80,82,83). Serial analysis of CTCs illustrates the molecular evolution of the primary tumor during the course of treatment. It may also have another advantage: even once the primary tumor is eradicated, CTCs continue to provide a 'real-time' non-invasive method of cancer cell genotyping.

CTC detection in peripheral blood is convenient, rapid and reproducible. Analyzing the characteristics of CTCs may help to evaluate the efficacy of therapy, provide a unique diagnostic resource and predict prognosis (84). Enumeration and identification of CTCs undergoing apoptosis may provide relevant information about responses to therapy in prostate cancer patients (75). Although the size of the tumor and the progress of the disease can be evaluated by radiography, its sensitivity is limited. The opportunity for tumor eradication is often lost due to the late detection of metastases by radiography (85). CTC monitoring is an early reproducible indication of disease status, superior to current imaging methods. Moreover, CTC levels appear to be superior to conventional imaging methods (even PET-CT) for evaluation of the response to treatment (86).

9. Concluding remarks

Since the phenotype and genotype of metastatic cancer cells are quite different from those of the primary tumor, CTC levels provide a more accurate method of evaluating the efficacy of chemotherapy and targeted therapy than analysis of the primary tumor (80). When significant CTC levels are confirmed, it may guide the treatment of patients who need adjuvant therapy, as a reasonable estimate can be made as to whether CTCs have been cleared from the peripheral blood. Tumor malignancy is associated with complex signaling pathways; therefore, it is desirable that CTCs be used as a tool to forecast prognosis, preferably in combination with another index, to comprehensively

monitor their clinical relevance (2,55). Detection, monitoring and molecular analysis of CTCs may provide a non-invasive approach to the detection of early tumor dissemination and the assessment of prognosis and appropriate treatment for established cancers (1). As more and more standardized and effective methods are established and the molecular mechanisms involved in metastasis are elucidated, and as more multi-center large sample clinical trials are validated, CTCs may be used as a real-time tool for the tailored treatment of cancer patients.

Acknowledgements

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