

Circulating Tumor Cells in Breast Cancer: Detection Systems, Molecular Characterization, and Future Challenges

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BACKGROUND: Circulating tumor cell (CTC) analysis is a promising new diagnostic field for estimating the risk for metastatic relapse and metastatic progression in patients with cancer.

CONTENT: Different analytical systems for CTC isolation and detection have been developed as immunocytochemical and molecular assays, most including separation steps by size or biological characteristics, such as expression of epithelial- or cancer-specific markers. Recent technical advancements in CTC detection and characterization include methods based on multiplex reverse-transcription quantitative PCR and approaches based on imaging and microfilter and microchip devices. New areas of research are directed toward developing novel assays for CTC molecular characterization. QC is an important issue for CTC analysis, and standardization of micrometastatic cell detection and characterization methodologies is important for the incorporation of CTCs into prospective clinical trials to test their clinical utility. The molecular characterization of CTCs can provide important information on the molecular and biological nature of these cells, such as the status of hormone receptors and epidermal and other growth factor receptor family members, and indications of stem-cell characteristics. This information is important for the identification of therapeutic targets and resistance mechanisms in CTCs as well as for the stratification of patients and real-time monitoring of systemic therapies.

SUMMARY: CTC analysis can be used as a liquid biopsy approach for prognostic and predictive purposes in breast and other cancers. In this review we focus on state-of-the-art technology platforms for CTC isolation, imaging, and detection; QC of CTC analysis; and

ongoing challenges for the molecular characterization of CTCs.

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The presence of circulating tumor cells (CTCs)² was first described in 1869 by Thomas Ashworth, an Australian physician (1). Twenty years later Steve Paget described in the first issue of *Lancet* “the seed and soil hypothesis,” according to which “metastasis depends on cross talk between selected cancer cells (the seed) and specific organ microenvironment (the soil),” a hypothesis revisited many years later by Fidler (2). Nowadays the cancer circulation problem is a very hot topic in cancer research (3).

The critical role that CTCs play in the metastatic spread of carcinomas is now widely recognized (4–6). European groups have clearly shown the clinical importance of disseminated tumor cells (DTC) in the bone marrow of breast cancer patients (7). CTC detection and enumeration in breast cancer have been established in several clinical studies, in which results showed a correlation with decreased progression-free survival and overall survival in operable breast cancer before (8–12) and after chemotherapy (13). In 2004 Cristofanilli et al. showed the importance of CTC detection for the estimation of disease progression and survival in metastatic breast cancer (14).

CTC analysis is a promising new diagnostic field for advanced-stage patients. However, because CTCs are very rare and the amount of available sample is very limited, such analysis presents formidable analytical and technical challenges. Recent technical advancements in CTC detection and characterization include

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² Nonstandard abbreviations: CTC, circulating tumor cell; DTC, disseminated tumor cell; RT-qPCR, reverse transcription quantitative PCR; CSC, cancer stem cells; EpCAM, epithelial cell adhesion molecule; ISET, isolation by size of epithelial tumor cells; SERS, surface-enhanced Raman spectroscopy; ICC, immunocytochemistry; FDA, US Food and Drug Administration; RT-PCR, reverse transcription PCR; CK, cytokeratin; DAPI, 4'-6-diamidino-2-phenylindole; HER2, human epidermal growth factor receptor 2; ALDH1, aldehyde dehydrogenase 1; MAM, mammaglobin; MUC1, mucin-1; EMT, epithelial-to-mesenchymal transition; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor.

multiplex reverse-transcription quantitative PCR (RT-qPCR)-based methods, image-based approaches, and microfilter and microchip devices. Highly analytically sensitive CTC detection platforms allow monitoring of disease and treatment efficacy (15).

CTCs are emerging tumor biomarkers, essentially providing a “liquid” biopsy sample and posttreatment monitoring while promising personalized treatment. Moreover, CTCs are well-defined targets for understanding tumor biology and tumor cell dissemination (16). Molecular characterization of CTCs offers an exciting approach to better understand the biology of metastasis and resistance to established therapies, and novel therapeutic targets may be identified by elucidating the relationship of CTCs to cancer stem cells (CSCs). Further research on the molecular characterization of CTCs should contribute to a better understanding of the biology of metastatic development in cancer patients.

In this review we focused on the presentation of state-of-the-art technology platforms for CTC imaging, detection, and molecular characterization and recent findings on the molecular characterization of CTCs and its future impact on the understanding of the metastatic process and personalized medicine.

Analytical Techniques for CTC Isolation, Detection, and Molecular Characterization

CTCs are extremely rare and as rare events follow the Poisson distribution (17). To reliably detect these cells, high assay efficiency and highly standardized preparation protocols are an absolute necessity. The Poisson distribution applies when randomly distributed objects (cells) are counted in a certain interval or volume (17). The limit of detection in the case of CTCs is not limited by addition of extra CTC identifiers or instrument improvement but by the amount of blood that can be examined for the presence of CTCs. This limitation must be taken into account before any analysis is started, especially in the case of early disease. A model that uses the Poisson distribution for blood collection and describes the statistics of the different processing steps that are needed for the isolation and detection of CTCs has been developed (17). In most cases CTCs are specifically detected by using a combination of 2 steps: isolation-enrichment and detection. Here we present the main analytical approaches currently used for CTC analysis (Table 1).

CTC ISOLATION AND ENRICHMENT

The most widely used approaches for the isolation and enrichment of CTCs involve density gradient centrifugation in the presence of ficol and immunomagnetic isolation. Both approaches are laborious procedures

with variable efficiency. These enrichment methods have also been combined, for example filtration devices with positive selection through an antibody against a pan-epithelial differentiation antigen, epithelial cell adhesion molecule (EpCAM), or ficol enrichment followed by positive immunomagnetic isolation. However, many studies have shown the heterogeneous nature of CTCs and indicated that all enrichment methods are biased because not all CTCs express the same cell-surface antigens, such as EpCAM (18–20). Microfluidic devices and filtration by size have also been recently developed. The main approaches for CTC isolation are presented in Fig. 1.

Immunomagnetic enrichment. Most assays to detect CTCs rely on positive selection based on EpCAM expression on tumor cells. These CTC enrichment technologies are based on anti-EpCAM antibody (18). However, some tumor cells express low or no EpCAM and in this way EpCAM-negative CTCs cannot be detected by immunomagnetic EpCAM-dependent enrichment methods. Evaluation of CTCs as prognostic markers should include EpCAM positive and negative subpopulations (19). Recently, it was reported that in contrast to other molecular breast cancer subtypes, “normal-like” cell lines lack EpCAM expression and are thus missed when CTCs are captured with EpCAM-based technology (20). Negative selection of CTCs has also been used through removal of leukocytes by anti-CD45 (15). Mostert et al. have recently proposed the combined use of anti-CD146 and anti-EpCAM to improve CTC detection in breast cancer patients (21), and Schindlbeck et al. have shown that the tumor cell detection rate was increased with the use of anti-CD176 (22). A highly sensitive and reproducible enrichment method that is based on binding to anti-CK alone or a combination of anti-CK and anti-EpCAM antibodies has been described. This method uses the Ariol® system (Genetix USA) for automated cell image capture and analysis of CTCs on glass slides (23).

Microfluidic and filtration devices for selection and enumeration of CTCs. A variety of state-of-the-art analytical platforms have been developed based on microfluidic and filtration devices for capture of CTCs. Here we describe only CTC filtration devices that have been evaluated in peripheral blood samples from cancer patients.

The isolation by size of epithelial tumor cells (ISET) system is based on the individual isolation of epithelial tumor cells by filtration because of their larger size compared to peripheral blood leukocytes. FISH was used to perform chromosomal analyses on tumor cells collected by using ISET, as well as PCR-based genetic analyses, which can be applied to ISET-isolated cells (24).

Table 1. Overview of analytical methodologies for the detection and molecular characterization of CTCs.

Assay system	Enrichment	Detection	Advantages	Disadvantages	References
Cytological assays					
CellSearch CTC test	Positive selection: EpCAM antibodies coupled on ferrofluids	Specific markers: CTCs: CK-19 Cell viability: DAPI	FDA cleared	EpCAM-positivity dependent	Riethdorf et al. (32)
CTC chip					
	Leukocytes: CD-45 Cell viability: DAPI		Visual confirmation of CTCs Clinical relevance in metastatic breast cancer	Limited number of markers	
	Positive selection: EpCAM antibodies coupled to microspots	Positive markers: CKs Negative marker: CD45	High detection rate Visual confirmation of CTCs	Subjective CTC analysis Further investigation on assay specificity	Nagrath et al. (27), Stott et al. (28)
EpiSpot assay					
	Negative selection: CD45 ⁺ cells	Nucleus: DAPI Immunological detection of secreted proteins: CK-19, mucin-1, cathepsin-D	Detects only viable cells	EpCAM-positivity dependent Clinical relevance not demonstrated	Alix-Panabières et al. (46)
Ariol system					
	CK antibodies and EpCAM antibodies coupled to microbeads	Positive markers: CKs Negative: CD-45 Nucleus: DAPI	High sensitivity Higher CTC detection rate than CellSearch	EpCAM- and CK-19-positivity dependent	Deng et al. (23)
CTC-filtering devices					
	Size (filters)	Positive markers: CKs Nucleus: DAPI	Capture and analysis platform	Tumor cells can be heterogeneous in size	Vona et al. (24), Lin et al. (25), Zheng et al. (26)
Laser-scanning cytometer					
	Positive selection: EpCAM antibodies coupled to columns	Fluorochrome-conjugated anti-epithelial antibody	Multiplexed imaging and genetic analysis Automated microscopic procedure for screening up to 5×10^4 cells in suitable time	EpCAM-positivity dependent	Pachmann et al. (31)
Dylight technology					
	Filtering device	Multiple antibodies: cytokeratin, HER2/neu, ALDH1, CD44, and CD24	Up to 10 000-fold enrichment Quantified minimal numbers of tumor cells	Application in cell lines	Balic et al. (33)
Molecular assays					
RT-PCR	Ficol gradient centrifugation	CK-19, HER2, h-MAM, ^a CEA, maspin, GABA A, B726P	High sensitivity	No morphological analysis	Slade et al. (35), Reinholz et al. (41) Continued on page 1245

Table 1. Overview of analytical methodologies for the detection and molecular characterization of CTCs. (Continued from page 1244)

Assay system	Enrichment	Detection	Advantages	Disadvantages	References
RT-qPCR	Ficol gradient centrifugation	CK-19, BST1, PTPRC	Prognostic significance in early breast cancer Detects only viable cells High sensitivity	No morphological analysis	Stathopoulou et al. (36), Stathopoulou et al. (37), Sieuwerds et al. (44)
RT-qPCR	OncoQuick enrichment and RNA preamplification	CCNE2, DKFZp762E1312, EMP2, MAL2, PPIC and SLC6A8, hMAM, and EpCAM	Quantification Detects only viable cells Not EpCAM dependent High sensitivity	No morphological analysis	Obermayr et al. (40)
Multiplex RT-PCR AdnaTest BreastCancer	EpCAM antibodies and MUC1 antibodies coupled to ferrofluidics	Multiplex PCR for: mucin-1, HER2, EpCAM, actin	Quantification Detects only viable cells High sensitivity	No morphological analysis	Aktas et al. (42)
Liquid bead array	Ficol gradient centrifugation and positive selection with EpCAM antibody-coupled ferrofluidics	Multiplex PCR for CK-19, HER2, MAGE-A3, hMAM, PBGD, TWIST-1	Detects only viable cells Saves sample and time, reduces cost	EpCAM and MUC1 positivity dependent assay No quantification	Markou et al. (45)
			Simultaneous detection of 6 markers on CTCs	EpCAM positivity-dependent assay	
			Detects only viable cells	End-point PCR	

^a hMAM, human MAM; CEA, carcinoembryonic antigen; GABA, γ -aminobutyric acid; PTPRC, protein tyrosine phosphatase, receptor type, C; CCNE2, cyclin E2; EMP2, epithelial membrane protein 2; MAL2, myelin and lymphocyte 2; PPIC, peptidylprolyl isomerase C; SLC6A8, solute carrier family 6 (neurotransmitter transporter, creatine), member 8; BST, bone marrow stromal cell antigen, MAGE, melanoma-associated antigen; PBGD, porphobilinogen deaminase.

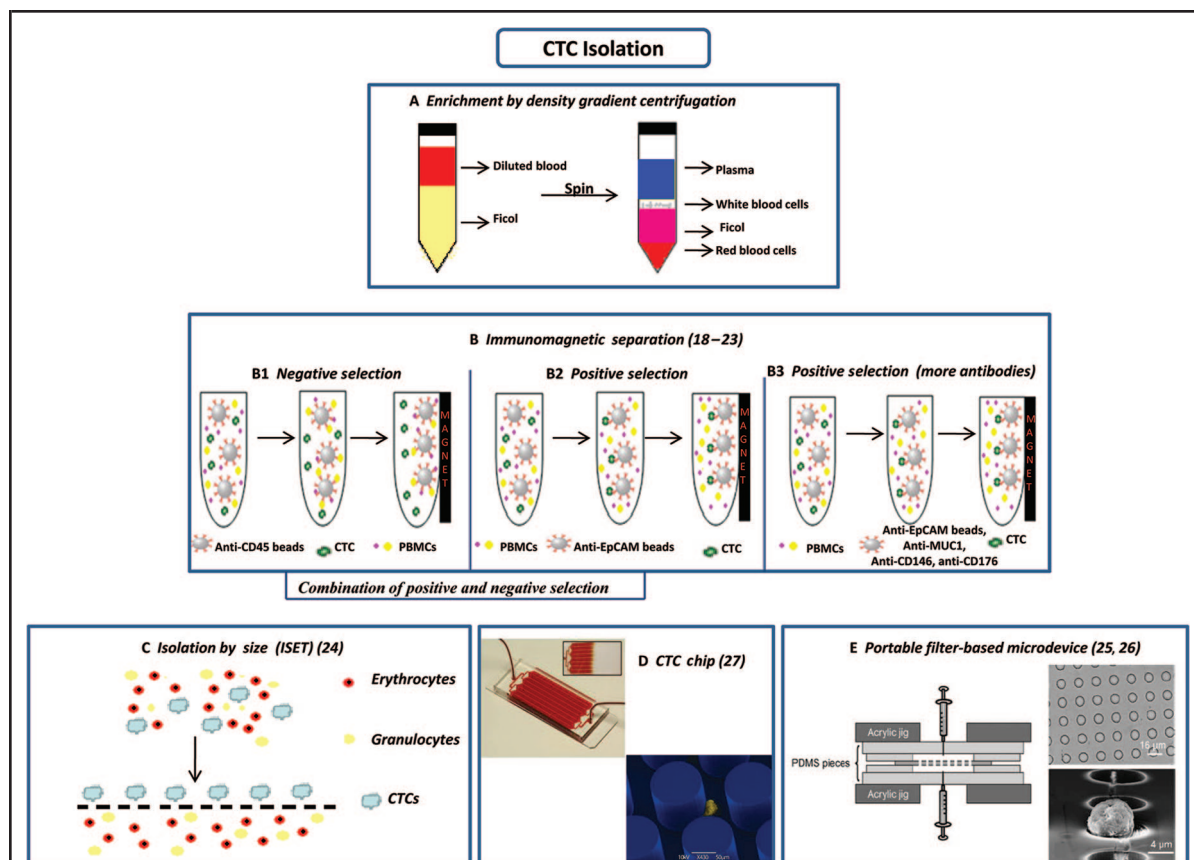


Fig. 1. Main approaches for CTC isolation-enrichment.

(A), Enrichment by density-gradient centrifugation in the presence of ficol. (B), Immunomagnetic separation [Fehm et al. (18), Königsberg et al. (19), Sieuwerts et al. (20), Mostert et al. (21), Schindlbeck et al. (22), Deng et al. (23)]. (B1), Negative selection through removal of leukocytes by anti-CD45; (B2), positive selection through an antibody against a pan-epithelial differentiation antigen, EpCAM; (B3), combined use of antibodies against CTC surface markers (anti-CD146, anti-CD176, anti-CK-19, and others). (C), ISET system [Vona et al. (24)]. (D), Microfluidic device: the CTC chip captures EpCAM-expressing cells in peripheral blood by use of anti-EpCAM-coated microposts [Nagrath et al. (27)]. (E), A portable filter-based microdevice filtration based on the size difference between CTCs and human blood cells [Lin et al. (25), Zheng et al. (26)]. PBMCs, peripheral blood mononuclear cells; PDMS, polydimethylsiloxane.

Lin et al. developed a portable filter-based microdevice that is both a capture and analysis platform capable of multiplexed imaging and genetic analysis and has the potential to enable routine CTC analysis in the clinical setting for the effective management of cancer patients (25). This device is based on the size difference between CTCs and human blood cells and has been reported to achieve CTC capture on filter with approximately 90% recovery within 10 min. The same group has developed and validated a novel 3-dimensional microfiltration device that can enrich viable circulating tumor cells from blood. The device provides a highly valuable tool for assessing and characterizing viable enriched circulating tumor cells in both research and clinical settings (26).

A microfluidic device, called the CTC chip, has been developed for capturing EpCAM-expressing cells in peripheral blood through the use of antibody-coated microposts (27). Lately, the same group has developed a high-throughput microfluidic mixing device, the herringbone chip, which provides an enhanced platform for CTC isolation (28). The design of the herringbone chip applies passive mixing of blood cells through the generation of microvortices to substantially increase the number of interactions between target CTCs and the antibody-coated chip surface. Efficient cell capture was validated by using defined numbers of cancer cells spiked into control blood, and clinical utility was demonstrated in specimens from patients with prostate cancer. The clinical util-

ity of these devices is currently under investigation. Another novel microfluidic device has been demonstrated that can selectively and specifically isolate exceedingly small numbers of CTCs through a monoclonal-antibody-mediated process by sampling large input volumes of whole blood directly in a short time period (29). The CTCs were concentrated into small volumes (190 nL), and the number of cells captured was read without labeling by use of an integrated conductivity sensor following release from the capture surface. The released CTCs were then enumerated on-device by using a novel, label-free solution conductivity route capable of detecting single tumor cells traveling through the detection electrodes. A new method has recently been developed that uses surface-enhanced Raman spectroscopy (SERS) to directly measure targeted CTCs in the presence of white blood cells (30). SERS nanoparticles with epidermal growth factor peptide as a targeting ligand have successfully identified CTCs in the peripheral blood of patients.

CTC DETECTION SYSTEMS

Recent technical advancements in CTC detection and characterization include: (a) image-based approaches like classic immunocytochemistry (ICC), the US Food and Drug Administration (FDA)-cleared CellSearch® system (Veridex), the Ariol system, and laser-scanning cytometry; (b) molecular assays based on nucleic acid analysis of CTCs, such as the highly sensitive RT-qPCR methods, multiplex reverse-transcription PCR (RT-PCR) assays, or a combination of molecular and imaging methods; and (c) protein-based assays like the EpiSpot assay, which detect tumor-specific proteins released by CTCs. Detection with anticytokeratin (anti-CK) antibodies is currently the most validated and standardized approach, and it also allows morphological interpretation of positive events.

These different approaches differ mainly in the way they detect CTCs (Fig. 2). Imaging approaches are based on the characterization of isolated cells as CTCs through fluorescently labeled antibodies mainly against epithelial antigens such as CK-19. A limited number of other protein markers have also been used for this reason. Conversely, molecular assays are based mainly on the analysis of gene expression in CTCs. By use of this approach the presence of a small number of CTCs can be shown through the highly sensitive detection of epithelial markers such as CK-19 in the presence of millions of peripheral blood mononuclear cells. Molecular assays cannot be used to accurately estimate the number of CTCs present in a sample; however, a plethora of molecular markers (e.g., gene expression, DNA mutations) can be detected in CTCs, making molecular characterization possible. These

approaches complement each other, because they give different information on CTCs.

Image-based approaches. Detection of disseminated tumor cells by use of classic ICC techniques, typically done by trained pathologists through visual observation of stained CK-positive epithelial CTCs, is time-consuming; it may take hours, if not days, if many samples are to be analyzed. Pachmann et al. have quantified minimal numbers of tumor cells by using the laser-scanning cytometer, a fast and quantitative automated microscopic procedure for screening that allows up to 10 000-fold enrichment (31).

The FDA-cleared CellSearch system (Veridex) (14) is based on a combination of ICC and immunofluorescence that uses specific markers for CTCs, such as CKs (mainly CK-19); leukocytes, such as CD45; and cell viability, such as 4'6-diamidino-2-phenylindole (DAPI) (nuclear stain) positivity. The CellSearch system for detecting circulating tumor cells has been validated via a rigorous clinical-testing program (32). This technology has produced the largest amount of clinical data on the prognostic relevance of CTCs in breast cancer. Recently, Sieuwerts et al. investigated whether the 5 subtypes of human breast cancer cells that have been defined by global gene expression profiling [normal-like, basal, human epidermal growth factor receptor 2 (HER2)-positive, and luminal A and B] were identified by CellSearch. These investigators found that the CellSearch isolation method, which uses EpCAM on the surface of circulating tumor cells for cell isolation, did not recognize, in particular, normal-like breast cancer cells that in general have aggressive features (20). Sieuwerts et al. concluded that new tests are needed that include antibodies that specifically recognize normal-like breast tumor cells but not cells of hematopoietic origin.

Balic et al. have developed a method for multi-marker image analysis for CTCs by employing novel DyLight technology (33). This imaging approach is based on the use of multiple antibodies [i.e., against CK, Her2/neu, aldehyde dehydrogenase 1 (ALDH1), CD44, and CD24] labeled with fluorochromes of different colors and spectral image analysis to separate different color spectra. This novel protocol will facilitate detection and phenotypical characterization of disseminated tumor cells, and with additional markers distinct subpopulations could be evaluated for the expression of particular therapeutic targets.

Molecular Assays for the Detection and Molecular Characterization of CTCs

Molecular assays for CTC detection and enumeration take advantage of the extreme analytical sensitivity and specificity of PCR. These assays are high

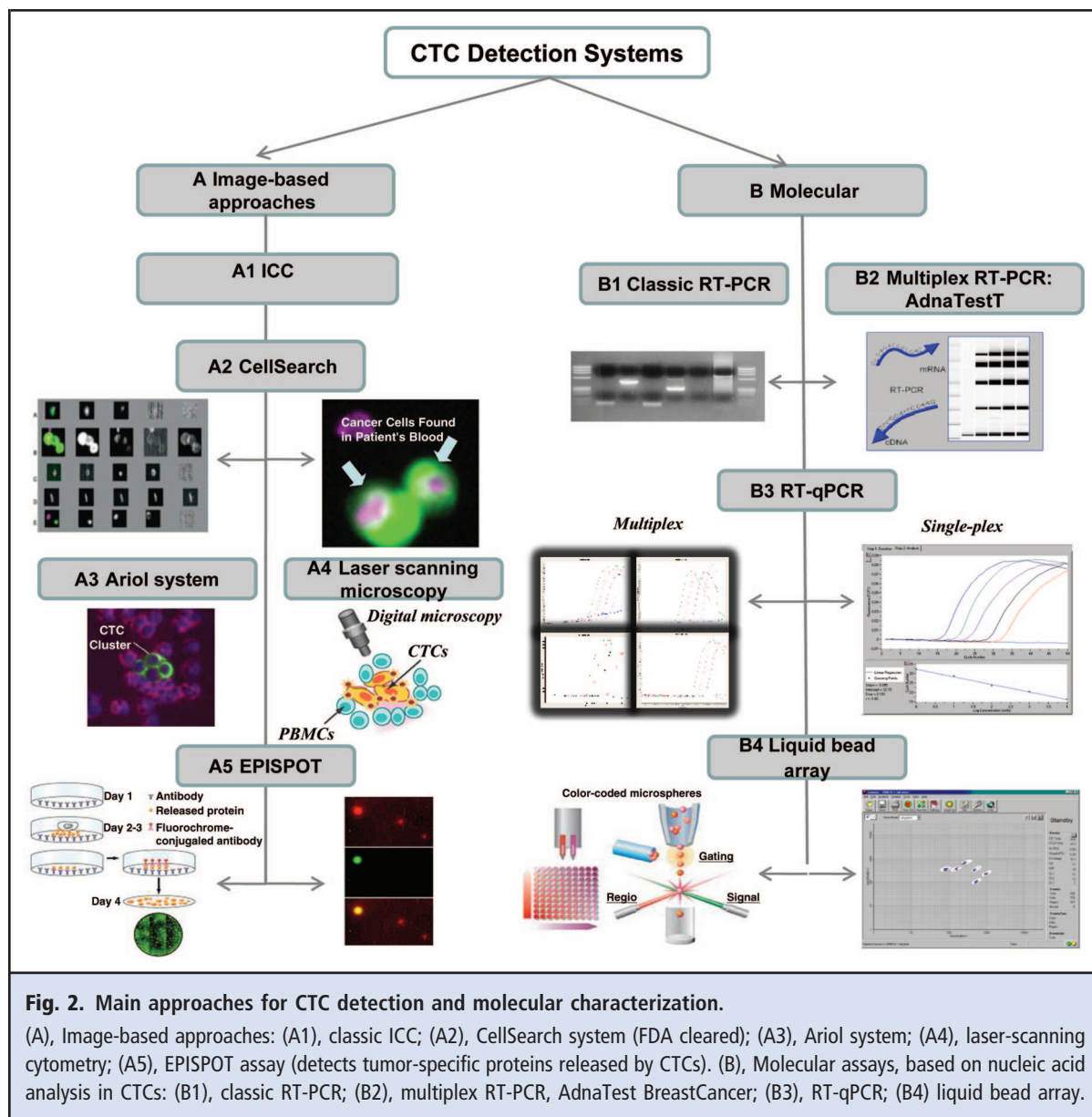


Fig. 2. Main approaches for CTC detection and molecular characterization.

(A), Image-based approaches: (A1), classic ICC; (A2), CellSearch system (FDA cleared); (A3), Ariol system; (A4), laser-scanning cytometry; (A5), EPISPOT assay (detects tumor-specific proteins released by CTCs). (B), Molecular assays, based on nucleic acid analysis in CTCs: (B1), classic RT-PCR; (B2), multiplex RT-PCR, AdnaTest BreastCancer; (B3), RT-qPCR; (B4) liquid bead array.

throughput and easy to perform because they are based on the isolation of total RNA from viable CTCs, and subsequent RT-PCR amplification of tumor- and epithelial-specific targets. Importantly, RT-qPCR assays can be designed in silico (through the use of specific software programs), easily automated, and subjected to internal and external QC systems (34).

Given the large background of circulating cells, it is probably necessary to detect 1 cancer cell in the presence of more than 10^6 leukocytes. Although RT-PCR is potentially analytically sensitive and specific enough to achieve this goal, success will require the use of appro-

appropriate mRNA markers. The only disadvantage of this approach is that it does not allow an accurate estimate of the number of CTCs present in a sample, because a different number of transcripts can be expressed from different cells, and only the number of target transcripts present can be estimated. A major advantage of molecular methods is their flexibility, especially to these multiplex assays, which reduces the required sample amounts, time, and analysis cost.

Almost 22 years ago Slade et al. developed an RT-PCR methodology for the detection of micrometastases in patients with breast cancer based on the estimation of the number of keratin 19 (*KRT19*, also known as

CK-19)³ transcripts in blood and bone marrow samples (35). Our group developed an RT-qPCR assay for *KRT19* mRNA (36, 37) and evaluated both its analytical and diagnostic sensitivity and its specificity and clinical potential for the molecular detection of occult carcinoma cells in peripheral blood of breast cancer patients (10–13).

Identification of specific subtypes of CTCs based on the expression of different genes can provide information about the biology of metastasis and improve patient management. To be effective, the method used to identify CTCs must detect all tumor cell types. However, the fact that CTCs are very rare and the amount of available sample is very limited presents tremendous analytical and technical challenges (38, 39). Using RT-qPCR, Obermayr et al. showed that a panel of 6 genes was superior to EpCAM and secretoglobin, family 2A, member 2 (*SCGB2A2*, also known as *mammaglobin* and *hMAM*) for the detection of CTCs in breast cancer, and that these genes may serve as potential markers for CTC derived from endometrial, cervical, and ovarian cancers as well (40). Reinholz et al. have shown that molecular characterization of circulating epithelial cells using *SCGB2A2* (*mammaglobin*) and B305D-C offers potential for early detection of invasive breast cancer (41). Recently, Aktas et al., using a commercially available kit (AdnaTest BreastCancer, AdnaGen AG), detected EpCAM, mucin-1 (MUC1), and HER2 transcripts in CTC and found that a major proportion of CTCs in metastatic breast cancer patients showed epithelial-to-mesenchymal transition (EMT) and tumor stem-cell characteristics (42). Interestingly, when estrogen receptor (ER) and progesterone receptor (PR) expression was assessed in CTCs by RT-PCR, the spread of CTCs was mostly found in triple-negative tumors, and CTCs in general were mostly found to be triple negative regardless of the ER, PR, and HER2 status of the primary tumor (43). A quantitative gene expression profiling method based on RT-qPCR to detect 1 CTC was performed by using a set of genes with no or minor expression by leukocytes (44). Several mRNA markers may be useful for RT-PCR-based detection of CTCs. Quantification of these mRNAs is essential to distinguish normal expression in blood from expression due to the presence of CTCs. Few markers provide adequate sensitivity individually, but combinations of markers may produce improved CTC detection. By using a multi-marker RT-PCR assay for CTC in early breast cancer, we

have shown that CTCs positive for *KRT19*; *SCGB2A2* (*mammaglobin*); and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (*ERBB2*, also known as *HER-2*) are associated with shorter disease-free survival (11).

We recently developed a multiplexed PCR-coupled liquid bead array to detect the expression of multiple genes in CTCs (45). With the use of this approach, 6 established CTC gene targets [*ERBB2*; *SCGB2A2* (*mammaglobin*); *KRT19*; melanoma antigen family A, 1 (directs expression of antigen MZ2-E) (*MAGEA1*); twist homolog 1 (*Drosophila*) (*TWIST-1*); and hydroxymethylbilane synthase (*HMBS*, also known as *PBGD*)] are simultaneously amplified and detected in the same reaction, in a very limited amount of CTC sample, thereby saving precious sample and reducing the costs and time of analysis. This assay forms an efficient basis for a multiplex approach to study the expression of up to 100 genes in CTCs.

Epispot assay. The EPISPOT (EPithelial ImmunoSPOT) assay, an adaptation of the ELISPOT assay (Autoimmun Diagnostica), was developed to detect tumor-specific proteins released by CTCs. According to the results of this assay, full-length CK-19 is released by viable epithelial tumor cells, and CK-19-releasing cells might constitute a biologically active subset of breast cancer cells with high metastatic properties (46).

QC in CTC-Detection Systems: Comparison of Different Methodologies

Clinical results of CTC analysis largely depend on the detection technology used. Despite the fact that most of these methods are analytically sensitive and specific, extensive studies have not been performed that were specifically designed to compare the efficacy of different detection methods when used to analyze the same clinical samples. This is an important issue for their clinical use because, particularly in early disease, differences in analytical sensitivity between these methods play a very critical role. Thus, standardization of micrometastatic cell detection and characterization is important for the incorporation of CTCs into prospective clinical trials to test their clinical utility. Results of numerous single-institution studies suggest that CTCs play an important role for risk stratification and monitoring of therapeutic efficacy. These findings must be evaluated in trials to verify the principle of this concept in the clinical setting.

The American Association for Cancer Research–FDA–National Cancer Institute Cancer Biomarkers Collaborative recently published a report on the validation and/or standardization of new biomarker tests

³ Human genes: *KRT19*, keratin 19; *SCGB2A2*, secretoglobin, family 2A, member 2, also known as *mammaglobin*; *ERBB2*, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian); *MAGEA1*, melanoma antigen family A, 1 (directs expression of antigen MZ2-E); *TWIST-1*, twist homolog 1 (*Drosophila*).

Table 2. Comparison studies between different laboratories and different methodologies for the same clinical samples.

Compared methods	Patients, n, type of breast cancer	Agreement	Reference
CellSearch vs Adnatest	245, Metastatic	50%	Fehm et al. (48)
CellSearch vs AdnaTest vs RT-PCR	79, Metastatic	CellSearch vs AdnaTest: 81%	Van der Auwera et al. (53)
		CellSearch vs RT-PCR: 57%	
		RT-PCR vs AdnaTest: 50%	
Oncoquick vs CellSearch	61, Metastatic	33/61 (54.1%)	Balic et al. (50)
RT-PCR vs ICC (DTCs compared)	385	280/385 (73%)	Becker et al. (52)
CellSearch	6 (14 independent laboratories)	Between-laboratory CVs 45%–65%	Kraan et al. (49)
		Between-instrument CV <20%	
		Between-assay CV <12%	
CellSearch	92 (3 independent laboratories)		Riethdorf et al. (32)

(47). The recommendations described within this report aim to accelerate the speed with which biomarkers can be used to fulfill their great promise to the personalized medicine revolution. From this point of view, agreement on the standardized detection of CTCs is absolutely necessary. Critical issues include: (a) the standardization of the preanalytical phase, such as sampling itself (e.g., sample volume, avoidance of cosampling of epidermal epithelial cells in case epithelial markers such as CK-19 will be used later for CTCs detection), sample shipping (stability of CTCs under different conditions), and storage conditions (use of preservatives, or anticoagulants); (b) standardization of CTC isolation through the use of spiking controls in peripheral blood; (c) standardization of detection systems; and (d) interlaboratory and intralaboratory comparison studies for the same samples. The development of international standards for CTC enumeration and characterization is also very important, especially in imaging detection systems that are observer dependent. In Table 2 we summarize comparison studies between different methodologies used for CTC detection.

In a recent comparison study by Fehm et al. between the CellSearch assay and a molecular test, the AdnaTest BreastCancer, concordant results regarding HER2 positivity were obtained in 50% of the patients (48). The authors concluded that a universal internal and external QC system for both CTC detection and enumeration is urgently needed before their application in the clinic. In another study, the feasibility of external quality assurance of the entire CellSearch procedure from blood draw to interpretation of results was

investigated by using samples from 6 cancer patients that were analyzed in 14 independent laboratories to test between-laboratory, between-assay, and between-instrument variation. In addition, between-operator variability was assessed through the interpretation of blinded images of all blood samples on a website. This multicenter study showed the feasibility of an external quality assurance program for CTC detection in patient samples and the importance of the continuation of such a program for the harmonization of CTC enumeration (49). For the QC of the FDA-cleared CellSearch system for metastatic breast cancer, a prospective multicenter study was conducted at 3 independent laboratories and involved samples from 92 patients with metastatic breast cancer. Intra- and interassay variation obtained by using controls containing defined numbers of cells, cell stability based on varying storage and shipment conditions, recovery precision from samples spiked with 4–12 tumor cells, interinstrument variability, and positivity of samples from metastatic breast cancer patients were tested (32). According to the results of this study, the CellSearch system enables the reliable detection of CTCs in blood and is suitable for the routine assessment in the clinical laboratory of blood samples from metastatic breast cancer patients. Blood samples should be shipped at room temperature, and CTC counts are stable for at least 72 h.

Balic et al. compared 2 methods for CTC enumeration, OncoQuick (Greiner Bio One) and the CellSearch system, and reported that the CellSearch system is more accurate and analytically sensitive than OncoQuick for enumeration of CTCs (50). Using spiked tumor cells, Punnoose et al. evaluated CTC capture

efficiency on different CTC technology platforms, including CellSearch and 2 biochip platforms, and used the isolated CTCs to develop and optimize assays for molecular characterization of CTCs. They reported similar performance in capturing CTCs for the various platforms tested, and found that capture efficiency is highly dependent on the level of EpCAM expression. According to the results of this study, addition of antibodies to mesenchymal markers could further improve CTC capture efficiency to enable routine biomarker analysis from CTCs (51).

RT-qPCR-based molecular methods can be used in the routine clinical laboratory because they can be standardized according to recently described quality issues such as quantification-cycle values, limits of detection, precision, and recovery (34). A direct comparison of detection rates of disseminated tumor cells in samples from a large cohort of 385 patients analyzed by use of both standardized standard ICC and real-time RT-PCR protocols showed a significant correlation between ICC and RT-PCR ($P < 0.01$), and the results of both methods agreed in 73% of cases (280/385) (52). In another recent study of methods for detecting CTCs, blood samples collected from 76 patients with metastatic breast cancer and from 20 healthy controls were in a direct comparison of 3 techniques: the CellSearch CTC System, the AdnaTest BreastCancer Select/Detect, and a previously developed real-time RT-qPCR assay for the detection of *CK-19* and *SCGB2A2* (*mammaglobin*) transcripts (53). Substantial variation was observed in the detection rates of CTCs in blood from breast cancer patients measured by using 3 different techniques. A higher rate of positive samples was observed with the use of a combined RT-qPCR approach for *KRT19* and *SCGB2A2* (*mammaglobin*), which suggests that this approach is currently the most analytically sensitive technique for detecting CTCs. Standardization of the AdnaTest BreastCancer kit and direct comparison with other established breast cancer CTC enrichment and detection techniques is still lacking, but greatly needed.

CTC Molecular Characterization and Individualized Cancer Treatment

Molecular characterization for CTCs can be used to increase our understanding of the biology of metastasis, to improve patient management, and to help identify novel targets for biological therapies aimed at preventing metastatic relapse. The role of CTCs in treatment failure and disease progression could be explained by their relation to biological processes, such as EMT and tumor dormancy (54). Identifying metastatic stem cells through molecular character-

ization approaches in the CTC population might result in the development of new therapeutic concepts.

CTCs are highly heterogeneous (4), as has already been shown through immunofluorescence (33), confocal laser-scanning microscopy (55), and molecular methods like RT-PCR (42, 55), RT-qPCR (44), and liquid bead array (45). This demonstrated heterogeneity is important, especially in cases in which therapeutic targets are expressed in CTCs and not in the primary tumor. However, the importance of CTC heterogeneity has not been fully exploited clinically as yet. Table 3 summarizes studies on the molecular characterization of CTCs in breast cancer.

HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR 2

HER2 analysis in CTCs may have clinical significance for HER2-targeted therapy because HER2-positive CTCs and disseminated tumor cells can be detected in patients with HER2-negative primary tumors who currently do not have access to HER2-targeted therapy (48, 56–63). There is a growing body of evidence that HER2 status can change during disease recurrence or progression in breast cancer patients. On the basis of this finding, reevaluation of HER2 status by assessment of HER2 expression on CTCs is considered to be a strategy with potential clinical applications (48, 56–63). A quantitative analysis by confocal microscopy assay for evaluation of HER2 expression in individual tumor cells has shown that there was a significant positive correlation between HER2 overexpression and gene amplification in individual CTCs (60).

HER2-positive CTCs have been detected in patients with HER2-negative tumors. Nevertheless, their presence is more common in women with HER2-positive disease (61). Such patients may benefit from (secondary) HER2-targeted therapy in an adjuvant setting. Therapy-resistant *KRT19* mRNA-positive cells in peripheral blood could be effectively targeted by trastuzumab administration (58). Moreover, the detection of *ERBB2* mRNA-positive CTCs after the completion of adjuvant chemotherapy may provide clinically useful information concerning the efficacy of treatment and the prognosis of patients with operable breast cancer (62). Changes of HER2 status in CTCs compared with the primary tumor during treatment for advanced breast cancer have also been reported (62, 63). Detection of *ERBB2* mRNA-positive CTCs after the completion of adjuvant chemotherapy may provide clinically useful information concerning the efficacy of treatment and the prognosis of patients with operable breast cancer (64).

Larger clinical trials are needed to evaluate the activity of HER2-targeted therapy in patients with acquired HER2 overexpression in CTCs. In a recent prospective multicenter trial, the HER2 status of CTCs in metastatic breast cancer patients was determined by

Table 3. Molecular characterization of CTCs.

Markers expressed in CTCs	Type of breast cancer	No. of patients (marker expressed, %)	Analytical methodology	Reference
HER2	Operable: after adjuvant chemotherapy	214 (21%)	Nested RT-PCR	Apostolaki et al. (62)
hMAM ^a	Operable	101 (13.9%)	Nested RT-PCR	Ntoulia et al. (67)
CK-19, hMAM, ^a HER2	Early	175 (CK-19: 41.1%; hMAM: 8%; HER2: 28.6%)	Multimarker RT-PCR	Ignatiadis et al. (11)
CK-19, HER2, MAGE-A3, hMAM, TWIST-1	Early	64 (CK-19: 26.6%; HER2: 12.5%; MAGE-A3: 18.7%; hMAM: 10.9%; TWIST-1: 31.2%)	Liquid bead array	Markou et al. (45)
CK-19	Early: after adjuvant chemotherapy	179 (41%)	Real-time RT-PCR	Xenidis et al. (13)
ER/PR	Early	48 (ER: 25%; PR: 4%)	RT-PCR	Fehm et al. (43)
HER2	Early	30 (33%)	Confocal microscopy assay	Cao et al. (60)
CK-19, HER2	Patients prior to chemotherapy	30 (CK-19: 33%; HER2: 83.3%)	RT-PCR	Bozionellou et al. (58)
HER2	Neoadjuvant	213 (24%)	CellSearch system	Riethdorf et al. (59)
HER2	Metastatic	45 (18%)	CellSearch system	Munzone et al. (64)
HER2	Metastatic	257 (50% CellSearch and 39% AdnaTest)	CellSearch system and AdnaTest	Fehm et al. (48)
EpCAM, MUC1, HER2, ER, PR	Metastatic	42 (EpCAM: 86%, MUC1: 86%, HER2: 32%, ER: 35%, PR: 12%)	AdnaTest	Tewes et al. (56)
VEGF, VEGF-2, HIF-1 α , pFAK	Metastatic	34 (VEGF: 62%, VEGF-2: 47%, HIF-1 α : 76%, pFAK: 92%)	Laser-scanning microscopy	Kallergi et al. (55)

^a hMAM, human MAM; MAGE, melanoma antigen; HIF, hypoxia-inducible factor; pFAK, phosphorylated–focal adhesion kinase.

comparing 2 CTC assays, both the FDA-cleared CellSearch assay and the AdnaTest BreastCancer (48). In this study, HER2-positive CTCs were detected in a relevant number of patients with HER2-negative primary tumors. Therefore, it will be mandatory to correlate the assay-dependent HER2 status of CTCs to the clinical response to HER2-targeted therapies.

ESTROGEN AND PROGESTERONE RECEPTORS

When the expression of ERs and PRs was assessed in CTCs by RT-PCR, Fehm et al. reported that, interestingly, the spread of CTCs was mostly found in triple-negative tumors, and that CTCs in general were mostly found to be triple negative regardless of the ER, PR, and HER2 status of the primary tumor (43). These investigators stated that (a) the clinical relevance may be different owing to the weak concordance between CTCs and disseminated tumor cells; (b) the biology of the primary tumor seems to direct the spread of CTCs; and (c) because the expression profile between CTCs and the primary tumor differs, the consequence for the selection of adjuvant treatment has to be evaluated.

ANGIOGENESIS MARKERS: EPIDERMAL GROWTH FACTOR RECEPTOR AND MAM

Using double-staining experiments and confocal laser-scanning microscopy, Kallergi et al. showed that the expression of pFAK (phosphorylated–focal adhesion kinase), HIF-1 α (hypoxia-inducible factor-1 α), vascular endothelial growth factor (VEGF) and VEGF2 in CTCs of patients with metastatic breast cancer could explain the metastatic potential of these cells and may provide a therapeutic target for their elimination (55). Studies of epidermal growth factor receptor (EGFR) expression in breast cancer have shown inconsistent results due in part to the large range of methods used. Anti-EGFR therapy trials have often not used patient selection because of this. Payne et al. used the CellSearch system to enumerate and measure EGFR expression on the surface of CTCs derived from the peripheral blood of individuals with metastatic breast cancer over time (65). Although proof for the clinical significance of EGFR-positive circulating tumor cells is currently lacking, expression of EGFR may predict response to lapatinib-based treatments as in a

case recently presented by Liu et al. (66). Mammaglobin expression has also been reported in CTCs (11, 41, 67).

CTCs, CSCs, AND EMT

The CSC hypothesis proposes that cancers arise in stem/progenitor cells through dysregulation of self-renewal pathways, generating tumors that are driven by a component of “tumor-initiating cells” retaining stem cell properties. A growing body of evidence indicates that subpopulations of CSCs drive and maintain many types of human malignancies (68). Therapeutic resistance, underlying tumor recurrence, and the lack of curative treatments in metastatic disease raise the question of whether conventional anticancer therapies target the right cells. Indeed, these treatments might miss CSCs that are resistant to many current cancer treatments, including chemotherapy and radiation therapy. These findings have important implications for the development and evaluation of oncologic therapies and present opportunities for potential gains in patient outcomes (68). One of the reasons CSCs are thought to escape antiproliferative chemotherapy is their relative dormancy (69).

Because CSCs survive standard cancer therapies and can theoretically regenerate the tumor even after the bulk of tumor cells are killed, novel treatment strategies may have to eliminate CSCs to be effective. Signaling pathways that maintain CSCs are attractive targets for these therapies. Korkaya et al. recently demonstrated that HER2 overexpression drives mammary carcinogenesis, tumor growth, and invasion through its effects on normal and malignant mammary stem cells (70). According to this study, overexpression of HER2 in a series of breast carcinoma cell lines increases the ALDH-expressing CSC population that displays increased expression of stem cell regulatory genes, increased invasion *in vitro*, and increased tumorigenesis in NOD/SCID (nonobese diabetic/severe combined immunodeficient) mice. The effects of HER2 overexpression on CSCs in breast cancer are blocked by trastuzumab in sensitive, but not resistant, cell lines, an effect mediated by the PI3-kinase/Akt pathway. These studies provide support for the CSC hypothesis because their results suggest that the effects of HER2 amplification on carcinogenesis, tumorigenesis, and invasion may be due to the effects of HER2 overexpression on normal and malignant mammary stem/progenitor cells. Furthermore, the clinical efficacy of trastuzumab may relate to its ability to target the CSC population in HER2-amplified tumors.

EMT phenomena endow epithelial cells with enhanced migratory and invasive potential, and as such have been implicated in many physiological and pathological processes requiring cell migration/invasion. Recently, it was shown that the induction of EMT not only allows cancer cells to disseminate from the pri-

mary tumor, but also promotes their self-renewal capability (71). Furthermore, the expression of stemness and EMT markers in CTCs is associated with resistance to conventional anticancer therapies and treatment failure, highlighting the urgency of improving tools for detecting and eliminating minimal residual disease (71). Although the relationships between EMT and CTCs remain largely unexplored, data have been recently reviewed that validate the implication of EMT processes in CTC formation and animal models with transplantable human breast tumor cells and help characterize EMT/CTC relationships (71).

Indeed, results of many different studies have shown that subsets of CTCs have a putative breast cancer stem-cell phenotype and express EMT markers. The first evidence of the existence of the putative stem-like phenotype within the disseminated tumor cells in bone marrow in early breast cancer patients was shown by Balic et al. (72). The expression of CSC markers such as CD44, CD24, and ALDH1 has also been shown in CTCs by both molecular assays (42) and imaging (73). Magnifico et al. have recently provided evidence for the therapeutic efficacy of trastuzumab in debulking and targeting tumor-initiating cells of HER2-overexpressing tumors. These authors also proposed that Notch signaling regulates HER2 expression, thereby representing a critical survival pathway of tumor-initiating cells (74).

Future Considerations

According to the parallel progression model recently proposed by Klein (75), parallel, independent progression of metastases arises from early disseminated tumor cells. Data from disease courses, tumor growth rates, autopsy studies, clinical trials, and molecular genetic analyses of primary and disseminated tumor cells are leading to the elucidation of a direct diagnostic pathology of systemic cancer (75). Molecular characterization of CTCs will provide important information for identification of therapeutic targets and understanding of resistance to therapies. Further research on the molecular characterization of CTCs will contribute to a better understanding of the biology of metastatic development in cancer patients.

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