Review Article

Methods for Detection of Circulating Tumour Cells and Their Clinical Value in Cancer Patients

(circulating tumour cells / disseminated tumour cells / clinical value / detection methods / breast cancer)

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Abstract. Currently available analytical methods enable identification, detection and characterization of circulating tumour cells in the peripheral blood and disseminated tumour cells in the bone marrow of breast cancer patients. About 0.01 % of the circulating tumour cells observed in the blood are able to form metastases. Therefore, they could be used for estimation of the risk for metastatic relapse, as a diagnostic tool for patient stratification, early determination of the therapy failure, or potential risk of resistance to the given therapeutic intervention. New therapeutic molecular targets could be identified for management of cancer patients using circulating tumour cell detection. The following review summarizes introduced methods of circulating tumour cell detection and their possible application in clinics.

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Abbreviations: BM – bone marrow, CECs – circulating epithelial cells, CEE – cell enrichment and extraction, CPCs – circulating progenitor cells, CTCs – circulating tumour cells, DTCs – disseminated tumour cells, EMT – epithelial-mesenchymal transition, ELISPOT – enzyme-linked immunospot assay, EPISPOT – EPIthelial immunoSPOT, ER – oestrogen receptor, ISET – isolation by size of epithelial tumour, LSC – laser scanning cytometry, MACS – magnetic cell sorting, MBC – metastatic breast cancer, MCRC – metastatic colorectal cancer, MEMS – microelectromechanical systems, MPC – metastatic prostate cancer, PA – photoacoustic, PB – peripheral blood, PR – progesterone receptor, WBC – white blood cells.

Introduction

Breast cancer is considered to be a systemic disease because early tumour cell dissemination may occur even with small tumours. Approximately 5 % of patients with breast cancer have clinically detectable metastases at the time of initial diagnosis, and further 30 % to 40 % of patients, who appear clinically free of metastases, harbour occult metastases (micrometastases), which are hidden and not detectable by the classic imaging and laboratory methods. Micrometastases can contribute to the disease relapse, and therefore their identification in cancer patients may have substantial effect on determining prognosis and individualizing treatment strategies for these patients (Clare et al., 1997; Braun et al., 2000).

The risk of metastasis development depends on multiple factors determined by overall tumour cell growth, survival, angiogenesis, and invasion. It is generally agreed that only a small and unique subset of cells from a primary tumour possess metastatic potential. Cells migrating from the primary tumour into the blood circulation are known as circulating tumour cells (CTCs). Detection of CTCs is a new field of cancer research focusing on detecting metastatic disease earlier, less invasively and more reliably than currently available conventional methods. Another group of tumour cells have been detected in the bone marrow of cancer patients. These cells are called disseminated tumour cells (DTCs). Both these cell types (CTCs and DTCs) could serve as "real-time biopsy" markers to monitor the tumour cell changes within the therapy.

Basically in all epithelial malignancies the disruption of epithelial cell homeostasis and the acquisition of a migratory mesenchymal phenotype, also described as epithelial-mesenchymal transition (EMT), allow these cells to travel from primary tumour to the site of metastasis formation without being affected by conventional treatment (Aktas et al., 2009). Circulating tumour cells do not represent a homogenous group, among these migrating cells also stem cell-like tumour cells have been

identified ($CD44^+/CD24^-$) as an active source of metastatic spread (Al-Hajj et al., 2003). Several abnormal circulating endothelial cell (CEC) and circulating progenitor cell (CPC) populations are present in cancer and their relationship to angiogenesis, apoptosis, vascular biology, and prognosis is unclear, but it is evident that CECs positively correlate with tumour invasiveness and size, possibly reflecting total tumour vascular volume (Goon et al., 2006, 2009). To reveal the importance of different tumour cell circulating populations in the metastatic process several immunoanalytical methods have been introduced into laboratory practice. Identification and counting of CTCs is methodologically limited by very low concentration of CTCs in peripheral blood (one CTC in 10^6–10^7 leukocytes). Enrichment is generally needed to increase sensitivity and it represents the initial step of the detection methods. The challenge of CTC detection is related to the requirement of high sensitivity combined with high specificity (Paterlini-Brechot and Benali, 2007). The following text offers a short overview of old and newly introduced CTC detection methods and their possible application in clinics.

Classification of the Methods

1. Imunocytochemical methods

The immunocytochemical detection of CTCs in peripheral blood (PB) or disseminated tumour cells (DTCs) in bone marrow (BM) can be considered as the gold standard method. The advantages of the immunocytochemical approach include the possibility to characterize and visualize by multiple staining, i.e., to combine immunostaining and fluorescence *in situ* hybridization analysis, together with morphological cell analysis (Riethdorf et al., 2008). Imunocytochemical detection of epithelial cells in PB or BM of breast cancer patients has been reported using various antibodies directed against epithelial membrane antigens (EpCAM, Ber-EP4, CK, MUC-1, CD44, CD24) or other cellular mucins, including tumour-associated glycoprotein-12 (Mansi et al., 1999; Pierga et al., 2004a).

DTCs are commonly detected by immunochemistry using epithelial antigens, such as cytokeratins (Pierga et al., 2004b; Krag et al., 2005; Woelfle et al., 2005; Hofmann et al., 2007). Detection of cancer-specific cytokeratins requires permeabilization of the target cell to enable staining of the intracellular compartment. The cell is losing its viability during the permeabilization along with the possibility to provide important information on the viability. Further, loss of cytokeratin expression may occur in cancer cells, which may result in false negative results. Conversely, false positivity is one of the main disadvantages of the immunocytochemical method. According to the antibody used, a false-positive detection rate of 1–3 % can be expected; the breast cancer cells show considerable heterogeneity in the expression of many carcinoma-associated cell surface molecules (Braun et al., 1999; Hofmann et al., 2007).

Immunocytochemical detection of DTCs/CTCs is labour-intensive, time-consuming and requires highly experienced investigator. Therefore, attempts to automate the screening procedures have been made, and many have proved to be successful. The use of new automated devices for the microscopic screening of large amounts of immunostained slides has already helped to increase the speed and reproducibility of immunocytochemical analyses (Bauer et al., 2000; Witzig et al., 2002; Kraeft et al., 2004). Imunocytochemical analysis is usually performed in combination with density gradient centrifugation, immunomagnetic procedures or size filtration methods to enrich tumour cells prior to their detection. Different separation techniques can be used to enrich CTCs specifically from complex biological mixtures such as cell and tissue homogenates, blood and other body fluids. The enrichment method can be based on morphologic cell characteristics (size or density) or on immunological characteristics.

Recently, a novel technique was described for DTC/CTC detection and characterization of single viable tumour cells. Epithelial immunospot assay (EPISPOT) is based on the secretion or active release of specific protein markers using an adaptation of the enzyme-linked immunospot (ELISPOT) technology. Using this method only viable tumour cells will be detected and appropriate protein secretion will be studied at the individual cell level, allowing direct determination of protein-secreting cell frequencies. The secretion enumeration of MUC-1 and CK-19 allowed the detection of viable DTCs in 90 % and 54 % of breast cancer patients with and without overt distant metastasis, respectively (Alix-Panabieres et al., 2009).

1.1. Size-based separation methods

The density gradient centrifugation standard separation technique allows the separation of mononuclear cells based on their lower density compared to other blood compartments. The fraction of mononuclear and tumour cells is separated from the blood cells and granulocytes using commercially available density gradient solutions (1077 g/ml): Ficoll (Amersham, Uppsala, Sweden), Lymphoprep (Nycomed, Oslo, Norway). Since whole blood quickly starts to mix with the density gradient, the system OncoQuick (Greiner BioOne, Frickenhausen, Germany) was developed. The Oncoquick system consists of a sterile 50 ml polypropylene tube with a porous barrier which is inserted above the density medium. This porous barrier prevents mixing whole blood with the separation medium. Up to 30 ml of anti-coagulated whole blood can be directly filled into the tubes. The increased tumour cell enrichment by Oncoquick provided an opportunity to further optimize tumour cell detection methods (Rosenberg et al., 2002; Baker et al., 2003).

Using the Isolation by Size of Epithelial Tumour (ISET) assay epithelial tumour cells can be isolated individually by filtration because of their larger size when compared to peripheral blood leukocytes. Peripheral

blood can be filtered through polycarbonate membrane with calibrated, 8 μM-diameter, cylindrical pores (Pinzani et al., 2006). The spots received after filtration (each corresponds to 1 ml of filtered blood) can be stained with cytological dyes (haematoxylin and eosin, May Grünwald-Giemsa, Reagena Ltd., Toivala, Finland) and/or characterized by immunolabelling, FISH, or TUNEL assays in order to analyse their antigens, aneuploidy and rate of apoptotic cells. The main advantage of ISET is its ability to isolate epithelial cells without damaging the cell morphology. The system has been shown to isolate one single tumour cell added by micropipetting to 1 ml of blood (Vona et al., 2000).

1.2. Antibody-based separation methods

Immunomagnetic cell separation methods are based on the interaction of magnetic particles conjugated to antibodies, which bind specifically to the targeted circulating tumour cells. When the mixed population of cells is placed in a magnetic field, those cells that have beads attached will be attracted to the magnet and may thus be separated from the unlabelled cells. Several types of beads are available, which are designed specifically for cell sorting. They differ in the size of beads and they require different strength of the magnetic field to separate the targeted cells. Of the larger beads (> 100 nm), the most commonly used type is the range produced by Dynal (Dynal Ltd., Wirral, Mersyside, UK). The smaller beads (< 100 nm) are represented by the MACS system produced by Miltenyi Biotech (Miltenyi Biotech Ltd., Church Lane, UK) and Rosette beads produced by StemCell Technologies (StemCell Technologies, Vancouver, Canada).

There are two major approaches to enrichment of a population with target cells, positive selection and negative depletion. In the positive selection procedure, target cells are immunomagnetically labelled, and the resulting enriched cell population is collected in the magnetically positive fraction. Conversely, negative depletion aims to immunomagnetically label non-target cells. Thus, an enriched target cell population is collected in the non-magnetic fraction. Currently there are several commercially available CTC tests on the market based on immunomagnetic separation.

Cellsearch system® (Veridex, Warren, NJ)

The CellSearch system represents the only Food and Drug Administration-approved system. This system consists of a CellSave sample tube for preserving and transporting blood samples, the CellSearch Epithelial cell kit containing reagents and consumables for performing the test, CellSearch control cells for assuring proper performance on a daily or run-to-run basis, an automated instrument AutoPrep for adding reagents and washing cells, and a semi-automated microscope for scanning and reading results CellSpotter Analyzer. The detection of CTCs via the CellSearch system is based on nanoparticles with magnetic core surrounded by a polymeric layer coated with antibodies targeting the EpCAM antigen for enrichment of CTCs. After immunomagnetic capture and enrichment, CTCs are conjugated with fluorescently labelled monoclonal antibodies predominantly against cell surface-specific antigens. The fluorescent antibodies include the following: anti-cytokeratin, which is characteristic for epithelial cell origin, DAPI, which stains the cell nucleus, and anti-CD45, which is specific for leukocytes. This mixture is inserted into a magnetic cell preservation device (MagNest, Immunicon Corp., Huntingdon Valley, PA), where magnetically labelled epithelial cells are attracted to the surface of a cartridge. The analyser automatically scans the entire surface of the cartridge. The cell is classified as a circulating tumour cell when it complies with two main requirements. First, the morphological features of the cell have to be consistent; second, the cell exhibits the $EpCAM^+$, CK^+, $DAPI^+$ and $CD45^-$ phenotype. It should be noted that the assay success is dependent upon the level of expression of the EpCAM and cytokeratin target antigens, which can vary significantly (Rao et al., 2005).

AdnaTest® (AdnaGen AG, Langenhagen, Germany)

Commercially available CTC detection – AdnaTest Breast, Colon, Prostate cancer system was developed for the immunomagnetic enrichment of CTCs from peripheral blood of cancer patients followed by detection of cancer-associated gene expression by reverse transcription and multiplex PCR (Schmitt and Foekens, 2009). This test enables the immunomagnetic enrichment of tumour cells via epithelial and tumour-associated antigens. Antibodies against epithelial and tumour-associated antigens are conjugated to magnetic beads (Dynabeads, Dynal International, Oslo, Norway) for the labelling of tumour cells in peripheral blood. The labelled cells are isolated by a magnetic particle concentrator and are subsequently lysed. The cell lysate is used for the isolation of mRNA via oligo$(dT)_{25}$-coated beads and further downstream gene expression analysis. The test has CE approval (Table 1).

Magnetic cell sorting (MACS)

Cells are labelled with a primary antibody specific to the cellular antigen and necessarily of the rat IgG class. Then, colloidal super-paramagnetic microbeads (goat, anti-rat IgG) are conjugated with the labelled cells. The cell suspension is loaded onto a wire-mesh separation column and placed into a strong magnetic field, where unbound cells are eluted as the "negative" fraction. The positive, labelled fractions of cells are eluted by demagnetizing the column. The MACS system has been used for EpCAM-positive isolation in several CTC studies (Miltenyi et al., 1990).

CEE Microfluidics®

Biocept (San Diego, CA) has developed the Cell Enrichment and Extraction™ (CEE) technology, which utilizes the company's proprietary system to capture rare cells in a microfluidic device (Fig. 1). Once captured, these cells remain suitable for molecular diagno-

Table 1. Description of tumour-associated markers used for detection of CTCs using AdnaGen® technology

AdnaTest	Abbreviation	Name	Function
Colon Cancer	GA 733-2	Epithelial Cell Adhesion Molecule (EpCAM)	EpCAM is a human cell surface glycoprotein over-expressed in epithelial cancers. EpCAM is involved in cell-to-cell adhesion. Over-expression of this gene appears to be associated with enhanced proliferation and malignant potential.
	CEA	Carcinoembryonic Antigen	CEA is a glycoprotein involved in cell adhesion. It is normally produced during foetal development. CEA measurement is mainly used as a tumour marker to identify recurrences after surgical resection in colorectal, gastric, pancreatic, lung and breast carcinoma.
	EGFR	Epidermal Growth Factor Receptor	EGFR is a member of the ErbB family of receptors. EGFR ligand binding induces the formation of homodimers and heterodimers and triggers the activation of downstream signalling pathways, such as the phosphoinositide-3 kinase (PI3K)/Akt pathway, which control cell proliferation, survival, and migration. EGFR is frequently over-expressed in breast cancer, and EGFR signalling is correlated with poor clinical outcome.
Breast Cancer	HER-2	Human Epidermal growth factor Receptor 2	HER-2 is a cell membrane surface-bound receptor tyrosine kinase and is normally involved in the signal transduction pathways leading to cell growth and differentiation. The *HER2* gene is a proto-oncogene. Approximately 15-20 % of BC display amplification of the *HER2*. HER over-expression and/or dysregulation may lead to increased/ uncontrolled proliferation, decreased apoptosis (programmed cell death), enhanced tumour cell motility, and angiogenesis.
	MUC-1	Mucin 1, cell surface associated	MUC-1 is highly expressed by the majority of human adenocarcinomas and is associated with poor prognosis. MUC-1 blocks intercellular adhesion.
	GA 733-2	Epithelial Cell Adhesion Molecule (EpCAM)	EpCAM is a human cell surface glycoprotein over-expressed in epithelial cancers. EpCAM is involved in cell-to-cell adhesion. Over-expression of this gene appears to be associated with enhanced proliferation and malignant potential.
Prostate Cancer	PSMA	Prostate-Specific Membrane Antigen	PSMA is a type II membrane protein with folate hydrolase activity produced by prostatic epithelium. The expression of PSMA in endothelial cells may be related to tumour angiogenesis and suggests a potential mechanism for specific targeting of tumour neovasculature. It is a possible therapeutic target for prostate cancer. PSMA is expressed on tumour cells as a non-covalent homodimer.
	PSA	Prostate-Specific Antigen	Prostate-specific antigen is a protein produced by the cells of the prostate gland. The level of PSA is often elevated in the presence of prostate cancer and in other prostate disorders.
	EGFR	Epidermal Growth Factor Receptor	EGFR is a member of the ErbB family of receptors. EGFR ligand binding induces the formation of homodimers and heterodimers and triggers the activation of downstream signalling pathways, such as the phosphoinositide-3 kinase (PI3K)/Akt pathway, which control cell proliferation, survival, and migration. EGFR is frequently over-expressed in breast cancer, and EGFR signalling is correlated with poor clinical outcome.
EMT-1/ Stem Cell	Akt-2	v-*akt* murine thymoma viral oncogene homologue 2	Akt-2 represents a subfamily of the serine/threonine protein kinases. Activation of Akt-2 is mediated by PI3K.
	Twist 1	Twist homologue 1 (transcription factor)	*Twist 1* gene binds to E-box elements on the *Akt2* promoter and enhances its transcriptional activity, and this is likely to be related to the EMT phenomenon in cancer cells.
	PI3Kα	Phosphoinositol 3-kinase α	PI3Kα activates the Akt-1 and Akt-2 Ser/Thr kinase, which is responsible for proliferation and anti-apoptotic function.
	ALDH1	Aldehyde dehydrogenase 1	ALDH1 is a new breast stem-cell marker. Over-expression of ALDH1 in the tissue of primary breast tumours correlates significantly with poor prognosis, and only ALDH1-positive cells are able to form metastasis in mice. In breast carcinomas, high ALDH1 activity identifies the tumorigenic cell fraction, capable of self-renewal and generation of tumours that recapitulate the heterogeneity of the parental tumour.

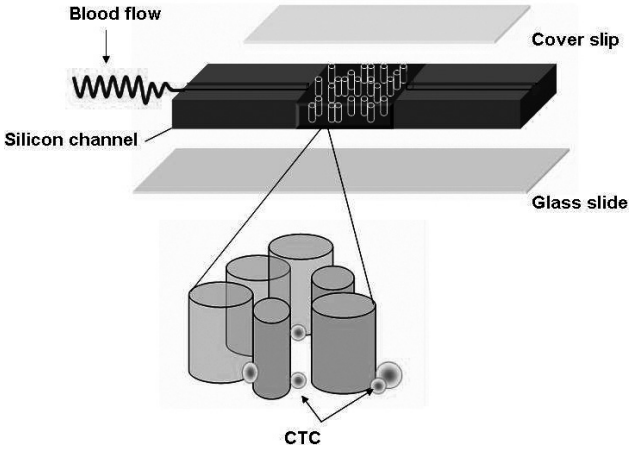

Fig. 1. CEE technology for enrichment of rare tumour cells

CEE™ enriches rare cells by combining antibody-functionalized surfaces with a microfluidics channel. Cells flowing through the CEE system are captured along a randomized array of functionalized posts coupled with specific antibodies which selectively attach to target cells. Captured cells can be used for further investigation.

sis either as intact cells or after lysis. Captured cells can be assessed morphologically, immunohistochemically, by fluorescent *in situ* hybridization (FISH) or by a variety of molecular analyses after extraction of the DNA or RNA. Placement of the posts and flow rates developed with mathematical models maximize cell capture in the microelectromechanical systems (MEMS) channel. Specified antibodies are coupled to the base and posts to selectively attach to target cells, creating an enriched cell sample. In breast cancer, cell-based tests such as FISH for *HER2/neu* oncogene are important, as is immunohistochemical staining. Such tests identify patients who will respond to oestrogen-based therapy. In colorectal cancer, specific chromosome abnormalities and changes in programmed cell death are well defined.

MagSweeper Technology®

The MagSweeper is an automated immunomagnetic separation technology that gently enriches circulating epithelial cells (CECs) from the blood 10^8-fold. The process of isolation of CECs is based on dilution of blood samples, which are pre-labelled with magnetic particles and loaded into the capture wells. The magnetic rods covered with plastic sheaths are swept through the well in concentric circular loops at a level of 1.5 mm above the bottom of the wells. After sweeping, magnets are washed in a circular loop to remove loosely bound contaminating cells. The rods are then immersed into a new buffer solution and disengage from the plastic covers. The external magnets located under the wells facilitate release of labelled cells and excess magnetic particles. MagSweeper can process 9 ml of blood per hour with 3–5 min total hands-on time, and is able to capture > 50 % of CECs as measured in spiking experiments without any significant changes in the gene expression of the captured cells. The ability to individually extract CECs provides opportunities for the detailed characterization of single cells for subpopulation studies of heterogeneous CECs (Talasaz et al., 2009).

MAINTRAC®

The MAINTRAC analysis allows quantitative detection of CECs, which is performed using Laser Scanning Cytometry (LSC) or image analysis with fluorochrome-conjugated anti-epithelial antibody (Lobodasch et al., 2007). Whole white cells from 1 ml of blood are incubated with HEA magnetic beads and FITC-conjugated mouse anti-human epithelial antibody. Labelled cells are separated on magnetic columns. First, negative cells are eluted from the column. Second, cells retained in the column are flushed out and unfixed vital cells are used for measurements applied to a poly-L-lysine-treated slide, which is subsequently analysed using LSC. LSC enables relocation of cells for visual examination of vital epithelial cells. This analysis allows detection of 1–2 cells per 10^7 after magnetic bead enrichment (Pachmann et al., 2005b). MAINTRAC analysis was used for monitoring the reduction in CEC numbers during the course of neo-adjuvant therapy in breast cancer patients. Strong correlation between the reduction in CTC numbers and chemotherapy has been shown and the final reduction in size of the tumour indicated that monitoring of CTCs would respond adequately to this therapy (Pachmann et al., 2005a).

2. Molecular based methods

The most commonly used molecular method for the detection of DTCs/CTCs relies on the screening of tumour-associated and/or organ-specific mRNA expression in cancer cells and on the absence of these gene products in the cells of the host tissue such as BM or PB.

The lack of unique DNA markers for breast cancer has induced the search for tumour-associated mRNA transcripts that can be amplified by the reverse transcription-PCR (RT-PCR) reaction (Pantel et al., 2003). It has been reported that RT-PCR is capable to detect one cancer cell per 10^6 normal cells. Quantitative real-time RT-PCR technology has been developed to increase the specificity of the RT-PCR approach (Mitas et al., 2001).

However, cancer cells show certain heterogeneity, and successful application of individual markers in the assay analysis is somewhat limited by its lack of specificity or sensitivity. Therefore, current study focuses on the multiple marker analysis, which may significantly improve sensitivity of detecting heterogeneous tumour cells.

A number of tumour-associated or epithelial-specific genes are used in the study to identify e.g. breast cancer cells, including *CK*, *Her2/neu*, *MUC1*, *hMAM*, *EpCAM*, *EGFR*, *hTERT*, survivin, *CD44*, c-*met* and several other mRNA markers (Stathopoulou et al., 2002; Alix-Panabieres et al., 2009; Botteri et al., 2009). The main advantage of the molecular approach is its sensitivity, which is considered to be higher than the reported sensitivity of immuno-mediated detection and immunocytochemistry.

Molecular profiling of CTCs may offer superior prognostic information with regard to the risk assessment for recurrence and predictive judgement of therapeutic regimens. The only validated multimarker assay for CTC molecular profile on the market is AdnaTest (AdnaGen AG, Langenhagen, Germany) – three tumour-associated antigens and one control gene are studied in standardized multiplex-PCR AdnaTest (for more details see Table 1), single assays are available for *CK19*, *hMAM* (Mitas et al., 2001; Stathopoulou et al., 2003, 2006; Xenidis et al., 2009). Additional testing is provided for EMT testing – three epithelial-mesenchymal markers, Twist-1, Akt-2, PI3Kα – and for tumour stem-cell marker ALDH1 (www.adnagen.com).

Non-invasive assay for detecting cancer®

The assay has been introduced as comparison between the phagocytic and non-phagocytic white blood cell (WBC) genomic profiles. Solid tumours shed 1–6 millions of CTCs into the blood per gram of tumour every day. Most of these cells undergo apoptosis and break into apoptotic bodies. Apoptotic bodies contain various intracellular molecules (e.g. DNA, RNA, proteins) and are cleared immediately from the circulation by phagocytic WBCs. Many genomic and proteomic signatures are unregulated in solid tumours. The comparison between non-phagocytic, phagocytic WBCs and tissue biopsy in patients and healthy controls could reveal them based on the difference in gene expression profiles detected by microarrays.

3. Physical principle-based methods

Hyperspectral microscope imaging system® – Quantitative immunoprofiling (CytoViva Technologies, Auburn, AL)

A new profiling system is comprised of a hyperspectral scanner, cocktails of engineered immunofluorophores, and analytic software applications. The scanning system uses a hyperspectral microscope imaging platform to measure spectral signatures over a specified bandwidth. Capture of complete emission spectrum from each fluorochrome and for each pixel avoids the information loss associated with narrow band filtering. The system's software utilizes curve-fitting algorithms to automatically match the acquired spectral profiles of scanner fluorophores with those in the spectral library. This allows identification and intensity measures of each fluoroconjugate in the specimen sample. The result is a biomarker expression map with information regarding spatial locations and relative intensities of each biomarker on any cell. Colour images allow user visualization. Quantitative immunoprofiling generates objective scoring results and makes possible reliable comparisons of data across samples and labs. Most importantly for researchers, these immunoprofile expression metrics can serve as phenotypic endpoints and molecular pathway attributes. The hyperspectral microscope imaging system is used to scan and analyse the pathology of tissue samples stained with four standard fluorochromes attached to specific antibodies. Quantitative values are recorded and can be compared with the visual interpretations.

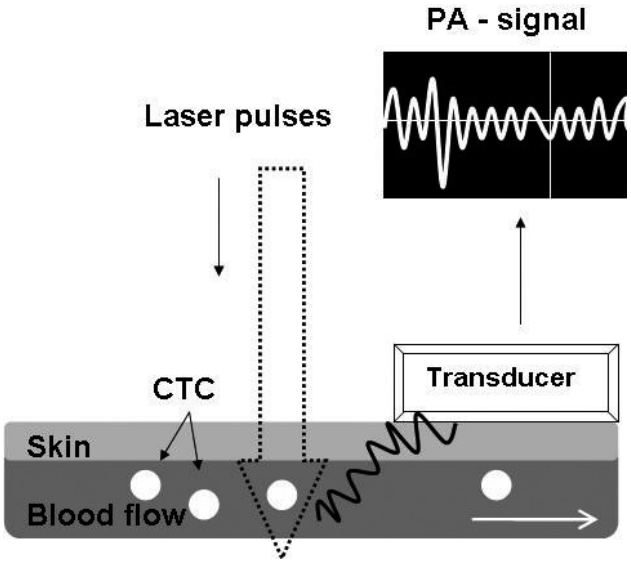

Fig. 2. In vivo multicolour photoacoustic flow cytometry
An ultraconic transducer on the skin surface detects acoustic waves generated by a laser beam interacting with a cell or a few cells.

In vivo multicolour photoacoustic (PA) flow cytometry

The ultrasensitive molecular detection of the CTCs was demonstrated on a mouse model of human breast cancer. Targeting of stem-like phenotype CTCs, which are naturally shed from parental tumours, has been performed with functionalized gold and magnetic nanoparticles. Magnet-induced clustering of magnetic nanoparticles in individual cells significantly amplified phototermal (PT) and photoacoustic (PA) signals (Fig. 2). The novel non-invasive platform, which integrates multispectral PA detection and PT therapy with a potential for multiplex targeting of many cancer biomarkers using multicolour nanoparticles, may prospectively solve grand challenges in cancer research for diagnosis and purging of yet undetectable tumour-initiating cells in circulation before they form metastasis. The cells coupled with gold nanoparticles could be eliminated by infrared laser photoablation non-invasively by hyperthermia (Zharov et al., 2007; Galanzha et al., 2009a, b).

4. Clinical impact of CTCs/DTCs

The prognostic and the predictive values of circulating tumour cells have been evaluated in several clinical studies, but discordant results have been reported. On the one hand this resides in the difference among the methods used for CTC identification, without clear definition of the criteria for CTC definition. Moreover, a high degree of variability in terms of stage of the disease, timing and number of blood samplings, clinical endpoint, statistical analysis, and insufficient follow-ups make the interpretation of clinical findings difficult.

The clinical relevance of CTCs in primary breast cancer is still under investigation. Detection rates of CTCs (e.g. numbers of CTCs) differ according to various approaches of researchers. The two widely used approaches to detect CTCs in peripheral blood of breast cancer patients are the FDA-approved assay CellSearch™ (Veridex) and the AdnaTest BreastCancer™ (AdnaGen AG). Clinical studies are focused mainly on assessing the clinical value of CTCs for therapy monitoring during the course of neo-adjuvant or adjuvant chemotherapy.

Positive detection of CTCs has been reported in primary breast cancer patients with no clinical signs of overt metastases in the range from 10 % to 60 % of patients (Witzig et al., 2002; Fehm et al., 2008; Alix-Panabieres et al., 2009). The presence of CTCs at the time of first diagnosis is an independent prognostic factor for overall and disease-free survival. Moreover, patients with CTC persistence after completion of systemic cytotoxic therapy are more likely to develop relapse compared with those who were CTC-negative. Targeting persistent CTCs is therefore an important issue to improve prognosis in primary breast cancer patients (Witzig et al., 2002; Alix-Panabieres et al., 2009).

The role of CTCs in primary breast cancer was studied in a prospective study involving 431 patients to assess the correlation between CTCs in blood and DTCs in BM, and to compare the expression profile of therapeutic relevant markers (HER-2, ER, PR) between CTCs and the primary tumour (Fehm et al., 2009). According to AdnaGen protocol the blood sample was regarded CTC-positive if at least one of three markers GA 733-2, MUC-1 or HER-2 was expressed. The detection rate of CTCs was 13 % and the presence of CTCs in patients significantly correlated with positive nodal status, negative ER and negative PR, respectively. The highest CTC positivity rate was obtained in triple-negative patients. Fehm et al. (2009) also evaluated the correlation of disseminated and circulating tumour cells with the result of weak association between CTC status and the presence of DTCs. The presence of CTCs was more closely related to the biology of the primary tumour than DTCs, but the expression profile of CTCs was different from the expression profile of primary tumour (Alix-Panabieres et al., 2009; Botteri et al., 2009; Fehm et al., 2009). Determination of the profiles of gene expression and/or proteins of tumours is becoming increasingly important since changes in the phenotype of the tumour cells can occur after the original diagnosis, and resistance to a treatment can only be inferred after the treatment has failed (Hayes et al., 2002; Alix-Panabieres et al., 2009; Botteri et al., 2009).

Few data are available evaluating the possible modification of CTC detection in the peri-operative period of patients undergoing surgery for operable breast cancer. However, this information may help our knowledge about the biology of the metastatic process and particularly about the impact of surgery on the release of cells into the bloodstream. The aims of the study with newly diagnosed breast cancer patients (Sandri et al., 2010a) were to evaluate whether there was an association between pathological variables and detection of CTCs, and to study the changes in CTC number between preoperative and postoperative samples. Blood samples from 69 patients were obtained before surgery and five days later during control visit. In case of positivity of at least one of the two peri-operative samples, a further blood sample was obtained on day 30. These samples were processed with the CellSearch system. Data from 56 patients remained for the final analysis. Sandri et al. (2010a) found that CTCs can be detected in the peripheral blood of approximately 30 % of patients undergoing surgery for primary breast cancer and that in these patients, vascular invasion is the only pathological characteristic associated with the presence of CTCs. Looking at the persistence of CTCs, Sandri et al. (2010a) found that only 25 % of patients were tested positive at the baseline and 5-day samples, and 10 patients in all were tested positive in one of the peri-operative samples and on day 30. This finding supports the idea about short persistence in the circulation because of fast removal of CTCs (Alix-Panabieres et al., 2009; Botteri et al., 2009; Sandri et al., 2010).

The second study represented by the SUCCESS trial is performed to evaluate the clinical value of CTCs in an adjuvant setting (Rack et al., 2008). So far, CTCs have been evaluated in 1,767 patients before adjuvant treatment. The positivity rate defined as the detection of more than one CTC was 10 % and after completion of cytotoxic therapy, 7 % of patients showed CTC persistence.

The clinical significance of CTCs in metastatic breast cancer has been clearly demonstrated. In a prospective, multicenter study 177 patients with measurable metastatic breast cancer levels of circulating tumour cells were tested both before the start of a new line of treatment and at the first follow-up visit (Kraeft et al.,. 2000). Results of this trial indicated that in metastatic breast cancer the level of CTCs before new therapy is elevated and, even more importantly, the level measured at the first follow-up visit is a useful predictor of progression-free survival and overall survival. The presence of five or more tumour cells in 7.5 ml of blood is associated with worse prognosis. Interestingly, the CTC count after the first cycle of chemotherapy indicates poor clinical outcome and the prognostic impact of increased CTC numbers is also maintained when repeated examinations during follow-up are performed. Moreover, CTC determination seems to be superior over conventional imaging methods for response evaluation (Kraeft et al., 2000).

The prospective multicenter studies in metastatic breast cancer (MBC), metastatic colorectal cancer (MCRC), and metastatic prostate cancer (MPC) demonstrated that the presence of CTC was a strong predictor of poor outcome (Miller et al., 2010). Using CellSearch system for MBC and MPC a threshold of 5 CTC/7.5 ml and for MCRC a threshold of 3 CTC/7.5 ml were used to stratify patients into those with favourable outcomes (CTC < 3 or < 5) and those with unfavourable outcomes (CTC ≥ 3 or ≥ 5). In all three cancers, patients with per-

Table 2. Summary of advantages and disadvantages of analytical methods for CTC detection and characterization

Analytical methods	Advantages	Disadvantages
Immunocytochemical methods	morphological analysis of CTCs/DTCs labelling of antigens on CTCs/DTCs quantification of CTCs/DTCs identification of CTCs/DTCs	time-consuming subjective evaluation
Epithelial immunospot (EPISPOT)	detection of viable cells detection of secreted proteins	proteins must be actively secreted or released no further identification and isolation of CTCs/DTCs
Immunomagnetic cell separation		
CellSearch system®	direct labelling of CTCs semi-automated fluorescent microscope evaluation enumeration of CTCs picture acquirement of CTCs FDA approved	no additional gene expression tests could be added for analysis of CTCs/DTCs subjective picture evaluation costly instrumentation
AdnaGen test®	recognition of tumour-associated markers isolated mRNA from CTCs can further be used for high-throughput gene expression profiling isolation and detection of stem cell and EMT markers low-cost instrumentation CE-diagnostic approval for the kit the CTC selection process and detection process could be used separately	enumeration of CTCs is not included false-positivity due to marker expression on non-tumour cells
MACS system®	smaller magnetic beads – better cell integrity ensured tumour cell enrichment obtaining intact cells or cell lysate suitable for further investigation (FISH, etc.)	not approved for CE so far
CEE Microfluidics®	combination of antibody-functionalized surfaces with microfluidics channel subsequent processing possible	capturing possible when cells make physiochemical contact with capturing surface relatively large volumes of the cells compared to the fluidic system
MagSweeper technology®	isolation of circulating epithelial cells keeps intact cell function does not perturb rare cell gene expression purified cells can be individually analysed	less uniform magnetic field gradient need for larger and more uniform capture region along the length of the magnet special laboratory instrumentation
MAINTRAC® analysis	analysis is possible from minute specimen minimally invasive fast, reliable and inexpensive	
Molecular based methods		
RT PCR	high sensitivity including several biomarkers (high-throughput)	illegitimate expression by normal cells (background expression) RNA instability visualization and enumeration of CTCs is not possible
Non-invasive assay for detecting cancer®	phagocytic and non-phagocytic WBC genomic profile comparison detected by microarrays larger amount of testing material	
Physical principle-based methods		
Hyperspectral microscope imaging system®	real-time optical imaging fast observation with unlabelled or fluorescently labelled cells	special laboratory device
In vivo multicolour photoacoustic flow cytometry	real-time counting of DTCs single cancer cell level performance	using endogenous chromophores or nanoparticles as photoacoustic contrast agents special instrumentation

sistent CTC counts had the worst outcome, which strongly suggested that their therapy was futile. Also patients that develop CTCs during the course of the therapy convert to a poor prognosis, similar to those with unfavourable CTCs before and after therapy (Miller et al., 2010).

In the study conducted at the European Institute of Oncology in Milan CTCs were analysed as a continuous

predictor to detect the shape of the relationship between CTCs and prognosis of MBC patients (Botteri et al., 2010). A total of 80 patients were enrolled into the study. Univariate analysis of clinicopathologic features associated with survival reported after a median follow-up of 28 months showed 76 progressions and 44 deaths due to breast cancer. Median survival was 33 months from the baseline. The number of CTCs at baseline, categorized as 0, 1–4, 5–20 and > 20, was significantly associated with progression-free survival (PFS) ($P = 0.03$) and overall survival (OS) ($P < 0.01$). The women with no CTCs showed a longer median PFS and OS compared to women with ≥ 1 CTC. A nonlinear increase in the risk of both progression and death with the increasing number of CTCs was observed, with a lessening increase after approximately 5 CTCs (Alix-Panabieres et al., 2009; Botteri et al., 2009).

In the prospective single institution trial (Tewes et al., 2009), molecular analysis of CTCs was performed in MBC patients and the technology developed by AdnaGen was used. The purpose of this study was to detect and analyse CTCs by molecular profiling during a follow-up of palliative chemo-, antibody- or hormonal therapy and determine the ability of this method to predict the response to breast cancer-related therapies. During the determination of the specificity for tumour cell selection and detection it was found that AdnaTest Breast Cancer has reasonably small false positive and false negative rates across a reasonable range of cut-off values. The statistical maximum for the diagnostic value was reached at a sensitivity of 80.5 % and a corresponding specificity of 80 %, respectively. The result for CTC detection in 42 patients with MBC at the time of tumour progression or therapy indicated the overall detection rate for CTCs to be 52 % with expression rates of 86 % for EpCAM, 86 % for MUC-1 and 32 % for HER-2. Comparison of antigen expression (ER, PR, HER-2) on the primary tumour with the expression on CTCs led to the findings that patients with ER, PR and HER-2-positive primary tumour in 45 %, 78 % and 60 % cases for ER, PR and HER-2, respectively, did not express these antigens on CTCs. Interestingly, 29 % patients with HER-2-negative primary tumours had HER-2-positive CTCs. The test predicted therapy response in 78 % of all cases. Persistence of CTCs significantly correlated with shorter overall survival. Based on these results molecular profiling of CTCs may offer superior prognostic information with regard to the risk assessment for recurrence and predictive judgement of therapeutic regimens (Alix-Panabieres et al., 2009; Botteri et al., 2009; Tewes et al., 2009).

The metastatic potential of a tumour is based on the presence of a low number of stem cell-like tumour cells that have been identified in tumour tissue to be an active source of metastatic spread. Furthermore, these cells may also undergo phenotypic changes, known as EMT. In the study involving 226 blood samples of metastatic breast cancer patients the expression of the stem cell marker ALDH1 and markers for EMT (AkT-2, Twist-1, PI3Kα) was determined (Aktas wet al., 2009). These findings correlated with the presence of CTCs and response to the therapy. Results indicated detection of CTCs in 31 % of all samples. In the CTC-positive group 81 % were positive for at least one of the EMT markers or ALDH1 or both. These findings correlated with the response to breast cancer-related therapies and indicate that a major proportion of CTCs show EMT and tumour stem cell characteristics, and these cells are an indication for therapy-resistant cell populations and thus for an inferior prognosis (Rao et al., 2005; Aktas et al., 2009).

Conclusion

Identification and detection of CTCs/DTCs could provide detailed insight into the biology of metastatic development in cancer patients. Based on these methods it has been possible to detect one tumour cell among one million other blood cells and further investigate its phenotype and genotype properties. Using these methods the epithelial-mesenchymal transition has been described. EMT is supposed to be the crucial mechanism of circulating tumour cell resistance against conventional treatment. Also circulating tumour cells with stem cell-like phenotype have been identified and are considered to be an active source of metastatic spread. Currently available drugs do not attack stem cell-like CTCs. This kind of research should provide new therapeutic targets and support the development of new anticancer therapies.

Molecular and functional characterization of CTCs/DTCs has shown phenotype heterogeneity of tumour cells not only among breast cancer patients, but even within the individual patient's tumour cell population. According to these findings detection and characterization of CTCs/DTCs can be used for estimation of patient prognosis in relation to the disease relapse and for stratification of patients to adjuvant therapy. CTCs/DTCs can be used as surrogate biomarkers for real-time monitoring of systemic therapy efficacy in individual patients supporting personalized therapy.

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