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Molecular characterization and phenotyping of circulating tumor cells for therapy optimization in metastatic breast cancer

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meiner Familie

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1 Introduction

"If you know your enemy and know yourself, you will win a hundred battles."

SUN TZU

1.1 What defines (breast) cancer?

Cancer is termed "malignant neoplasm" or "malignant tumor" and is defined as a group of diseases that is characterized by a rapid generation of abnormal cells with the potential to grow beyond their usual boundaries (**"metastasis"**, see chapter **1.2**) [1]. There is a widespread consensus that most cancer types are monoclonal growths descended from single normal progenitor cells [2]. More than 100 types of cancer have been classified so far, whereby the tissue of origin specifies the respective cancer characteristics. Cancers that develop from epithelial cells -such as breast cancer- are designated carcinomas and make up approx. 85% of all cancer types [3]. The transformation of a normal cell into an abnormal one, meaning the progression from a pre-cancerous lesion to cancer, requires the interplay of multiple aspects. That is, the individual genetic background, the person's age as well as extrinsic biological (e.g. viruses, bacteria), chemical (e.g. tobacco smoke, aflatoxin) and physical (e.g. ultraviolet/ionizing radiation) factors [1]. Cancer accounted for 8.2 million deaths in 2012 and thereby is among the leading causes of morbidity and mortality worldwide. Within the next two decades, cancer cases are expected to rise from 14 (in 2012) to 22 million [1, 4].

1.1.1 Traits of cancer

According to Hanahan and Weinberg [5] cancer can be defined by the following characteristics (*hallmarks*) (**Figure 1-1**): sustaining proliferative signaling, evasion of growth inhibitory signals, resistance against apoptosis (programmed cell death), replicative immortality, formation of new blood vessels (angiogenesis) and an invasive/metastatic potential. Further attributes that might be involved in cancer pathogenesis include deregulation of the cellular metabolism, evasion of the immune system, development of genomic instability/mutability and finally, tumor-promoting inflammatory actions that are triggered by the innate immune system [5].

Figure 1-1: Hallmarks and characteristics of cancer. In 2000, six hallmark capabilities (autonomous proliferative signaling, evading growth suppressors, resisting apoptosis, inducing angiogenesis, enabling replicative immortality and activating invasion and metastasis) have been proposed. The illustration further encompasses so-called next generation hallmarks and further characteristics involved in cancer pathogenesis, respectively: genomic instability, metabolic deregulation, inflammation and escaping immune destruction. **Modified after [5].**

Within the presented study, the main emphasis was to investigate the cancer hallmark "invasion and metastasis" (marked in red in **Figure 1-1**), especially with regard to the characterization of circulating tumor cells (CTCs) involved in this process. Both aspects are comprehensively described in chapters **1.2** and **1.3**.

1.1.2 Breast cancer

1.1.2.1 Epidemiology and etiology

Worldwide, breast cancer is the most frequently occurring cancer among women, with approximately 1.7 million women diagnosed and 522,000 cancer-related deaths registered in 2012 [6]. According to the American Cancer Society, one in eight U.S. women will develop invasive breast cancer over the course of her lifetime [7]. Further, the international incidence of female breast cancer is estimated to reach about 3.2 million new cases per year by 2050 [8]. With 70,000 new cases and nearly 18,000 deaths in 2011, breast cancer is also by far the most common cancer in women in Germany [9]. At the time of diagnosis, approx. 25%

of all affected women are younger than 55 years and one in 10 is under the age of 45; the median age at onset is 64. For female patients in Germany, the five-year relative survival rate is given with 87% [9, 10].

Risk factors for developing breast cancer can be divided into lifestyle-related factors and factors that are not related to one's personal choice. The former include extrinsic factors as discussed above, as well as alcohol abuse, obesity, physical inactivity, hormone replacement therapy, being nulliparous or late-bearing. Factors that cannot be influenced are, e.g. gender, age, ethnicity, family and personal history, early menarche/late menopause and density of the breast tissue [10]. About 5-10% of all breast cancers occur due to inherited genetic dispositions, whereby mutations in the tumor suppressor genes *BRCA1* and *BRCA2* (breast cancer 1/2, early onset) are the most common ones. Regarding *BRCA1/2*, genetically predisposed women have an estimated lifetime risk of 50-80% for breast cancer [11, 12].

1.1.2.2 Breast cancer classification

The female breast is composed of small mammary glands that produce milk (*lobules*), tubes that transfer the milk from the glands to the nipple (*ducts*), and stromal tissue (fat, blood/lymphatic vessels, connective tissue) surrounding it (**Figure 1-2A**). Breast cancers can arise from different areas of the breast, whereas most of them originate in the glandular tissue. Based on their morphological characteristics, they can be classified into lobular, ductal, medullary, mucinous, adenoid cystic, inflammatory and tubular carcinomas [8]. Therein, the ductal carcinomas are the most frequent ones: they form in the lining of a milk duct (intraductal) and either are non-/pre-invasive (ductal carcinoma *in situ*, DCIS) or have already infiltrated the surrounding stromal tissue (invasive ductal carcinoma, IDC) (**Figure 1-2B**). IDC accounts for about 75% of all reported breast cancer cases, followed by invasive lobular carcinoma (ILC, 10%) deriving from the milk-producing lobules [13].

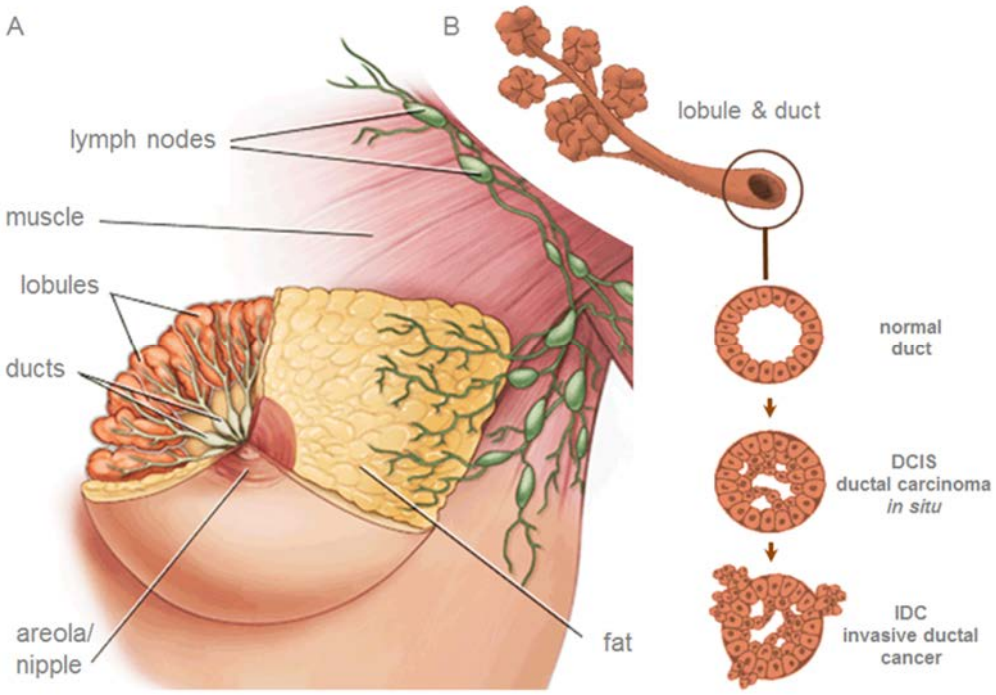

Figure 1-2: Female breast anatomy and breast cancers arising from the duct. The female breast (A) is a network of lobules (small milk producing glands) and ducts (tubes transporting milk from lobules to the nipple). Lobules and ducts are organized in grape-like patterns ('lobes', not shown) and are surrounded by fatty tissue. Lymphatic vessels (green) of the breast mainly lead to the axillary (under arm) lymph nodes. The most common type of breast cancer begins in the cells of the duct (B): DCIS or ductal carcinoma *in situ* originate from the lining of the milk duct and can progress to invasive cancer (IDC) spreading into the fat tissue. **Modified after [14] and [15].**

Besides the histopathology, breast cancers can be further classified by their grade and stage. The ***grading*** is based on the appearance of cancer cells compared to normal cells and on how rapidly cancer cells are dividing. Thus, a low grade (G1) indicates that the tumor is growing slowly, is less likely to spread and consists of cells that are well differentiated. Grade 2 (G2) describes a moderately differentiated tumor, whereby poorly differentiated and fast-growing tumors are graded 'G3'. For pathological ***staging*** the American Joint Committee on Cancer (AJCC) TNM system is commonly applied. Therein, the T ('tumor') followed by numbers 0-4 describes the tumor size and spreading to tissues near the breast, N ('node') followed by 0-3 indicates whether and to which extent lymph nodes are affected, and M ('metastasis') followed by 0 or 1 gives information if the cancer has spread to distant organs (e.g. lungs/bone). In general, a larger tumor, lymph node spread and metastasis are associated with higher stage numbers and a worse prognosis [16].

1.1.2.3 Breast cancer heterogeneity and treatment

Breast cancer is regarded as heterogeneous disease comprising a high degree of inter- and intratumoral diversity. Hereby, several tumor-intrinsic and -extrinsic features such as the

aforementioned histological, as well as immunopathological, transcriptional, genomic, epigenetic, and (micro)environmental aspects contribute to breast tumor heterogeneity. Differences in breast tumor groups/subtypes are associated with distinct clinical behavior, courses of the disease and treatment responses [13].

Asides from structural heterogeneity, high variability in protein and gene expression patterns can be observed. The presence of specific markers helps to define subtypes and identifies tumors that are susceptible to targeted therapies [13]. Proteins that are assessed by immunohistochemistry include the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2). Additionally, HER2 gene amplification is determined via fluorescence *in situ* hybridization (FISH).

Breast cancer subtypes and treatment strategies

Further molecular signatures can be defined by testing gene expression panels (e.g. PAM50, Oncotype DX®, MammaPrint™, EndoPredict®) [17]. Thus, tumors can be grouped into two ER/PR-positive (ER^{pos} and/or PR^{pos}; *luminal A and luminal B*) and three ER-negative (ER^{neg}) subtypes, and the *triple negative* ($ER^{neg}/PR^{neg}/HER2^{neg}$) group. ER-positive tumors are generally smaller, low grade and node-negative, while ER-negative tumors are less common but have a worse prognosis. One ER-negative group shows elevated HER2 expression (*HER2 subtype*), another high gene expression of rather myoepithelial/basal-like cells (*basal-like subtype*), and a third harbors a varied gene expression profile (*normal-like subtype*) [8]. Besides intertumor heterogeneity, these variable subtypes can also be the explanation for intratumoral heterogeneity, where different degrees of basal-like and luminal features occur, originating either from less differentiated stem cell-like or luminal lineage-committed progenitor cells [18].

The following treatment strategies are available: *endocrine therapy*, *chemotherapy* and *targeted therapy*. Luminal ER^{pos}/PR^{pos} tumors grow hormone-dependently and can be treated by hormone receptor modulators (e.g. tamoxifen), blocking of the estrogen production (e.g. aromatase inhibitors; letrozole/anastrozole) or with an ER-degrading agent (fulvestrant) [19]. Cytotoxic chemotherapy is particularly beneficial with respect to ER^{neg} tumors, wherein it can be administered in different regimens/combinations (e.g. cyclophosphamide/doxorubicin). Targeted therapies comprise small-molecule compounds or monoclonal antibodies directed towards molecules needed for carcinogenesis. Among them are tyrosine kinase inhibitors (e.g. against HER2, c-Met), inhibitors of angiogenesis and intracellular signaling pathways (ERK, mTOR, PI3K/AKT), and substances that interfere with DNA repair mechanisms (e.g. PARP inhibitors) [19]. For instance,

implemented in the standard of care in HER2-overexpressing breast cancer patients are trastuzumab, a monoclonal antibody that blocks the HER2 receptor, and lapatinib, a tyrosine kinase inhibitor interfering with HER2/EGF (epidermal growth factor) receptor pathways.

Resistance to HER2 targeting therapy

However, all therapies are challenging due to the occurrence of primary and acquired resistance. Resistance mechanisms can be manifold ranging from impaired uptake mechanisms to improved DNA damage repair (reviewed in [20]). Taking the upper example of HER2-overexpressing breast cancer patients, resistance to trastuzumab/ lapatinib could be shown with regard to *PI3K* (phosphatidylinositol-3-kinase) gene alterations leading to its hyperactivity and thereby to dysregulation of various cellular processes (e.g. cell growth, proliferation, differentiation) [21, 22]. Furthermore, it has been reported that PI3K hotspot mutations within its *PIK3CA* (PI3K catalytic subunit alpha) gene encoding the catalytic subunit may be discordant between the primary tumor and its corresponding metastases potentially conferring therapy resistance [23]. This discrepancy can also be seen when phenotypes of the primary tumor sites are compared with the seed for metastasis, the circulating tumor cells (CTCs) – with potential implications for cancer treatment strategies [24].

Developing CTC-based treatment strategies

Currently, breast cancer medical treatment is adjusted to the traits of the prevailing primary tumor phenotype making it quite challenging to match an appropriate and effective therapy with each patient's need. Because of the phenotypic differences between primary tumor and CTCs several clinical trials are run to use the CTCs' features to streamline and tailor-cut cancer management (see chapter **1.3.3**). In particular, to investigate whether patients with $HER2^{neg}$ primary tumors but $HER2^{pos}$ CTCs benefit from HER2-targeted therapies is aimed by large multi-center clinical trials, the DETECT studies. Therein, the DETECT III study (NCT01619111) was one of the first trials in which the treatment choice is based on phenotypic characteristics of CTCs. Moreover, a translational project of the DETECT III intends to analyze the presence of activating *PIK3CA* mutations in $HER2^{pos}$ CTCs using the SNaPshot assay [25] (see **manuscript 3.1.1**).

1.2 What defines metastasis?

Metastasis describes a complex multi-stage process by which cancer cells leave the original primary tumor site and migrate to other parts of the body. Consequently, metastasis is the difference between benign and malignant growth and represents the major clinical problem of cancer [3]. Despite significant improvements that were made in diagnostics, local and systemic adjuvant therapies and also in surgical technologies, most cancer deaths are due to metastases that resist or are not eradicated by conventional therapies [26]. It is estimated that at least half of the patients suffer from metastatic disease at the time of cancer diagnosis [27] and 90% of cancer deaths are a consequence of these metastases [28].

1.2.1 The metastatic cascade

Metastasis is the result of a complex process of sequential events that is often termed as metastatic cascade [26]. Broadly, this cascade can be divided into three main steps: invasion, intravasation and extravasation (**Figure 1-3**). More precisely, these events can be complemented by: cell migration before local invasion, dissemination of tumor cells after they entered the blood circulation, arrest at secondary sites, colonization/engraftment at distant sites upon extravasation, and finally, formation of micro- and clinically detectable macrometastases [29, 30].

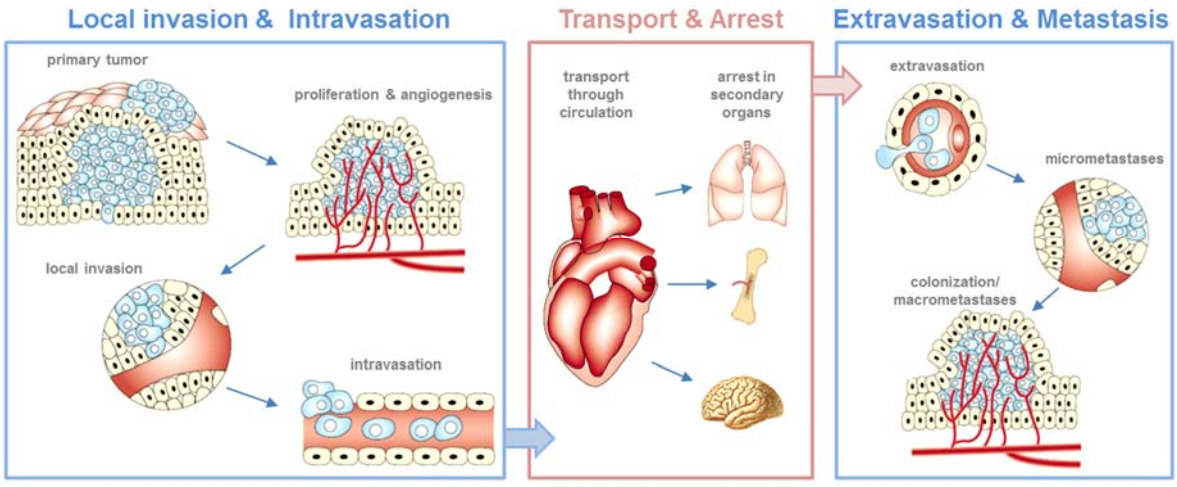

Figure 1-3: The main steps of the metastatic cascade. In the initial step, the *in situ* primary carcinoma proliferates, which is accompanied by extensive blood vessel formation (angiogenesis). Thereafter, local invasion of the stroma (extracellular matrix, ECM) by some tumor cells occurs through breaching the basement membrane. Tumor cells may then intravasate into either lymphatic or blood microvessels where the circulation transports these cells to distant anatomical sites (e.g. lung, bone, brain). Cancer cells that survive in the circulation become trapped in the capillary beds of distant organs by adhering to endothelial cells or the subendothelial basement membrane. Some may extravasate to form (dormant) micrometastases, which then can acquire the ability to colonize the distant tissue in which they have landed. Upon successful colonization, clinically detectable macrometastases may develop from these founder cells. **Modified after [26] and [2].**

1.2.1.1 Invasion and intravasation

The travel of tumor cells from their origin to other anatomical sites starts with the loss of cell-cell adhesion capacity and local invasion of the tumor-surrounding stroma (**epithelial-mesenchymal transition**, see **1.2.2**) [31]. In order to break free from the cellular cohesion, various mediators such as cell adhesion molecules (CAMs, e.g. E-cadherin), integrins and proteases (e.g. matrix-metalloproteinases, MMPs) have to orchestrate in enabling cancer cell motility [3]. Consequently, the basement membrane and the extracellular matrix (ECM) are degraded and remodeled to create new space for cancer cells. Further matrix-modifying and cell stimulating substances such as MMP-9, VEGF (vascular endothelial growth factor), EGF, and CSF-1 (colony stimulating factor 1) are released ensuring tumor cell invasion and the transport of nutrients upon new vessel formation (=angiogenesis) [2, 32]. Blood vessels within close vicinity to the tumor may then provide a route for the entry of cancer cells to the circulatory system [31]. Similarly, the process of intravasation (penetration of a cell into a vessel) requires supportive stromal cells including macrophages and endothelial cells which assemble to guide cancer cells on their way to the lumen of capillaries [33].

1.2.1.2 Transport through the circulation

Cancer cells that have entered the blood flow are designated as **circulating tumor cells** (**CTCs**, see chapter **1.3**). They are transported singly, in cancer cell clusters and/or escorted by blood components (e.g. platelets) [34]. Since blood with its hydrodynamic shear forces represents a rather hostile compartment for CTCs, the interplay with platelets can be regarded as protective entourage in the circulation [2]. However, this presumed protection makes the passage through narrow microvessels even more complicated. The internal diameters of most capillaries (3-8 µm) are too small to pass. Erythrocytes with a size of only approx. 7 µm and their deformability can easily pass, while cancer cells with approx. 20 µm will get stuck. Due to their larger size, CTCs become trapped in small vessels (arterioles) within the pulmonary capillary beds [2]. But if CTCs pass the lungs and reach the arterial circulation, they can be dispersed to all tissues of the body. According to the concept of the “first-pass organ” (first organ in flow direction downstream from the primary tumor), the layout of the circulation may also influence the favored site of metastasis (see **1.2.1.3**) [2, 3].

1.2.1.3 Extravasation and colonization

Extravasation describes a process reverse to intravasation: the escape of a cancer cell from the blood or lymphatic vessels to invade the tissue parenchyma of distant sites.

Extravasation depends on critical interactions with the walls of the vessel in which they have become trapped [2, 35]. This so-called transendothelial migration of cancer cells requires a “docking” (arrest/adhesion to endothelium) and a “locking” (enhanced cell contact with numerous focal adhesions) step as it has been reported for the extravasation of leukocytes (diapedesis). Highly specific cell adhesion molecule profiles (e.g. selectins, immunoglobulins, integrins), cell clustering and a local inflammatory response are all together inevitable to achieve efficient extravasation [35]. Cancer cells can extravasate singly or upon already started proliferation at the vessel wall. For the latter, Crissman *et al.* provided a detailed morphological study proposing distinct sequential extravasation events: a metastasizing CTC that has been physically trapped in a capillary gets surrounded by platelets building up a microthrombus. Within this thrombus, the cancer cell forms cytoplasmic extensions and pushes aside an endothelial cell allowing a direct contact to the underlying capillary basement membrane. Afterwards, the thrombus is removed by proteases and the cancer cell starts to proliferate intravascularily. Finally, the subendothelial matrix gets disrupted and cancer cells can break through [36].

Upon arrival at secondary sites, cancer cells may form small micrometastases (<2 mm) - representing the targets for adjuvant therapies -, and then eventually develop into detectable macrometastases (≥2 mm). Generally, metastatic progression can be regarded as a function of space and time. One striking feature of metastasis is the capability of different tumor types to colonize/metastasize at organ-specific sites (“organ tropism”), wherein the range of targeted tissues broadly varies. Already in 1889, Stephen Paget postulated that seeding at secondary sites is not a random process. According to his “seed and soil” hypothesis the interaction of cancer cells (“seeds”) with their microenvironment (“soil”) plays a crucial role in metastasis formation [26, 37]. For example, breast cancer metastasis typically occurs in bone, lung, liver and brain, whereas metastatic relapse of prostate cancer is mainly confined to bone [38]. Another entity-specific aspect is “metastatic latency” referring to the time elapsed between the diagnosis of the primary tumor and the outgrowth of detectable metastatic lesions [30, 38]: breast cancer recurrence is often detected after years or decades of remission [39]. By contrast, lung cancers were shown to establish distant metastases within months of primary diagnosis [40]. Consequently, the ability of cancer cells to infiltrate distant organs does not always come along with their competence to develop metastases. Regarding breast cancer, a large pooled analysis revealed that more than 30% of the patients showed up with hundreds and thousands of micrometastases in their bone marrow at the time of initial diagnosis, but only half of them evolved metastatic disease [41].

Colonization, the last step of the invasion-metastasis-cascade, can be seen as the most inefficient process of all. The main bottlenecks are, for instance, cellular stress passing through endothelial barriers, the lack of survival supporting signals and supportive stroma (“soil”), and hostile cells of the innate immune system [30]. However, unraveling underlying mechanisms of cancer cell elimination and critical determinants of this process, respectively, have yet to be realized [30, 42].

The herein portrayed description of metastasis underpins the so-called linear progression model, which localizes selection of genetic/epigenetic alterations mostly inside the primary tumor: CTCs with primary tumor characteristics evade from the primary site to colonize distant secondary sites [42]. However, more recent data propose that parallel dissemination occurs (parallel progression model): CTCs depart the primary lesion before a fully malignant phenotype has been acquired; somatic mutations and metastatic growth subsequently develop at distant sites [43].

1.2.2 Epithelial-mesenchymal transition (EMT)

Cellular mechanisms that confer tumor cells the ability to disseminate from the primary tumor (invasion/intravasation, transport) and to resist apoptosis include a series of dynamic conversions of epithelial to mesenchymal cell states (epithelial-mesenchymal transition, EMT) and its reverse process, mesenchymal-epithelial transition (MET).

In general, EMT can be classified into three different subtypes depending on the biological context. First, it was recognized as a fundamental regulatory program of normal development involved in embryogenesis including gastrulation, neural crest formation and myogenesis (=type 1). Further, EMT has been shown to occur during processes such as wound healing and organ fibrosis (=type 2) and finally, it is associated with cancer progression and metastasis (=type 3) [44-48]. While the common outcome of all three classes of EMTs, which represent distinct biological settings, is the generation of motile cells of mesenchymal phenotype triggered by shared genetic features, the mechanisms of EMT induction as well as progression vary tremendously from one EMT type to another [47].

1.2.2.1 Traits of epithelial and mesenchymal cells

During EMT, a cell of epithelial origin undergoes a series of phenotypic changes thereby gaining mesenchymal features including motile and invasive characteristics. Epithelial cells exhibit a columnar/polygonal shape with apico-basal polarity, are tightly connected by

intercellular adhesion complexes (tight and adherens junctions, desmosomes, gap junctions, integrins) and are adjoined to a basement membrane at their basal surface. Identified markers characteristic for the epithelial state are, for instance, epithelial cadherin (E-cadherin) as basolateral and mucin 1 (MUC1) as apical marker, different cytokeratins (8, 9, 18), claudins, occludins and desmoplakin [49]. On the contrary, mesenchymal cells are spindle-shaped and lack any cell polarity as well as connective adhesions allowing their individual movement through the extracellular matrix [50]. Typical mesenchymal markers are neural cadherin (N-cadherin), vimentin, fibronectin, β-catenin and vitronectin [49] (**Figure 1-4**).

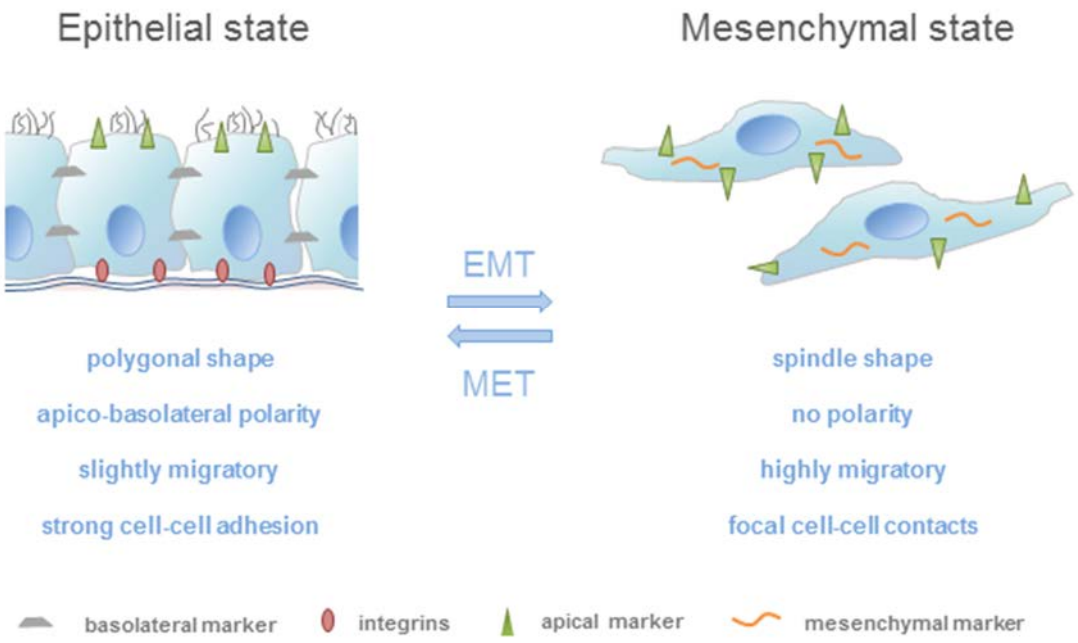

Figure 1-4: Characteristics of epithelial and mesenchymal cell traits. EMT: epithelial-mesenchymal-transition; MET: mesenchymal-epithelial transition. **Illustration based on descriptions in [49-51].**

1.2.2.2 The EMT program

A number of extracellular triggers and pathway crosstalks are required for EMT initiation and progression entailing the disassembly of inter- and intracellular adhesion structures [50]. EMT-activators include - among others - components of the extracellular matrix (e.g. collagen, hyaluronic acid) as well as soluble growth factors such as fibroblast growth factor (FGF), EGF, transforming growth factor-β (TGF-β) and hepatocyte growth factor (HGF). In response to these ligands, effector molecules of the small GTPase (Rho, Ras, Rac) and Src tyrosine-kinase families get activated [49] which then activate downstream transcriptional regulators such as SNAI1, SNAI2, TWIST and zinc finger E-box binding homeobox 1 (ZEB1) mediating changes in EMT-related gene expression patterns [49]. The

major impact of all extracellular stimuli is the repression of E-cadherin either directly (by SNAI1, SNAI2, ZEB1) or indirectly (by TWIST), resulting in its cleavage/degradation and thereby disruption of homotypic adherens junctions [52, 53].

1.2.2.3 EMT in (breast) cancer

Activation of the EMT program in (circulating) tumor cells paves the way for cancer progression by stimulating proinvasive processes, producing reactive stromal cells and by mediating the cancer stem cell phenotype [44]. Yu *et al.* investigated EMT in CTCs with regard to metastatic breast cancer and found that a significant number of CTCs exhibited a partial or complete EMT phenotype, whereby their presence was associated with disease progression [54]. Furthermore, it has been reported that EMT preferentially occurs in a specific molecular breast cancer subtype: cells with a basal-like (stem cell) phenotype seem to be more prone to undergo EMT than other subtypes, accompanied by a more invasive/aggressive behavior [55]. Concerning cancer, EMT not only contributes to tumor invasion, but could also be causative for resistance to/escape from cell death, senescence, chemotherapy, immune surveillance, and might confer stem cell capabilities [46].

1.3 What defines circulating tumor cells?

The first description of CTCs is attributed to Thomas R. Ashworth who reported in 1869 about “a case of cancer in which cells similar to those in the tumours were seen in the blood after death” [56]. CTCs are cancer cells that have detached the primary tumor site or metastatic deposits and have been shed (“mobile CTCs”) or actively invaded (“motile CTCs”) the blood circulation [57] (**Figure 1-5**).

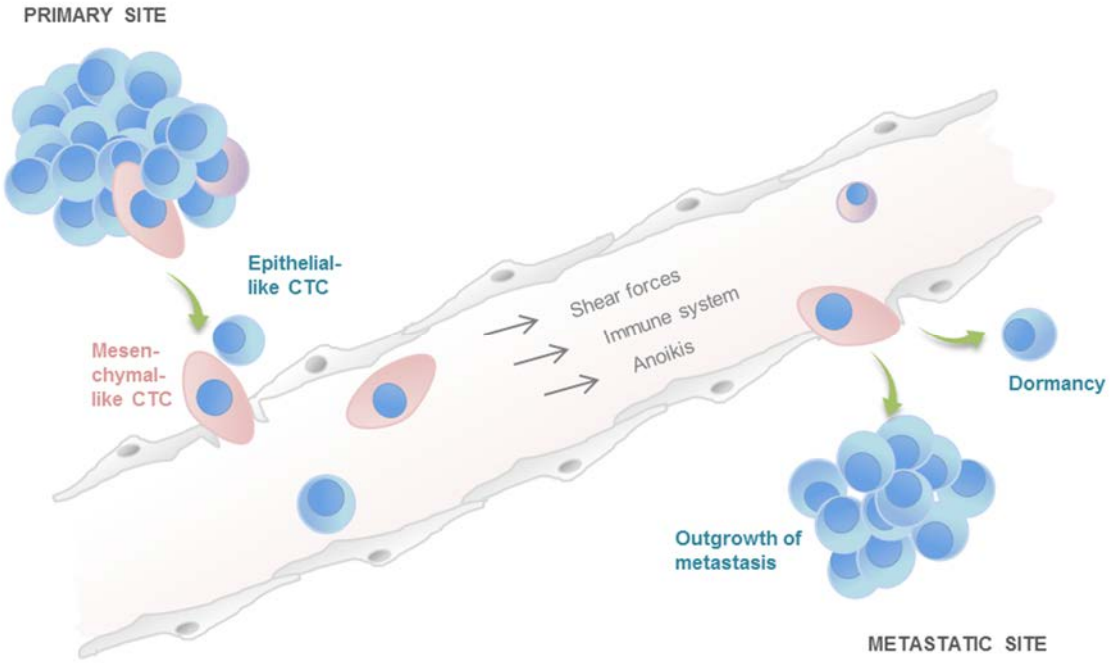

Figure 1-5: Fates of CTCs during invasion and metastasis. Tumor cells with different molecular profiles (epithelial-like, mesenchymal-like) lose adhesion to neighbouring cells in the primary tumor and invade the surrounding stroma; then CTCs passively or actively enter the lymphatic or vascular system (via EMT, centrosome amplification). Within the circulation, CTCs have to resist shear forces, escape from the immune system and avoid anoikis. Cells that overcome these hurdles might extravasate and transmigrate into secondary organs as disseminated tumor cells (DTCs); some remain dormant and persist to initiate secondary metastatic lesions and subsequently may start seeding to tertiary sites.

It is assumed that 1 g of tumor mass (approx. 10^9 cells) releases about $3x10^6$ cells per day into the blood stream [58]. However, most of these cells cannot cope with the rather hostile environment that they are exposed to or they die due to first-pass effects, resulting in persistence of a very limited number of CTCs within the circulation. For breast cancer patients the maximum CTC half-life is estimated to be 0.5 to 2.4 hours [59]. Typically, in 1 ml of blood 1 to 10 CTCs can be found in the background of supernumerary blood cell components, composed of $6x10^6$ leukocytes, $2x10^8$ platelets and $4x10^9$ erythrocytes [60, 61].

1.3.1 Criteria for CTC determination

According to the FDA (Food and Drug Administration)-cleared CellSearch® Circulating Tumor Cell Test (Janssen Diagnostics, LLC, US) a CTC phenotype is defined by the criteria depicted in **Figure 1-6**. Besides enrichment via EpCAM (Epithelial Cell Adhesion Molecule), a CTC stains positively for multiple epithelial cytokeratins (CK) (predominantly CK8, 18 and 19)[1] and negatively for CD45 (cluster of differentiation 45). It displays a nuclear DAPI (4',6-diamidino-2-phenylindole) stain and ideally is of oval to round shape with at least 4 µm in size. However, also elongated, irregular shaped and multi-nucleated events may count as CTC [62-64]. Further, it is known that cellular and nuclear sizes as well as nuclear-to-cytoplasmic ratios may vary broadly, even among cells from the same individual [62]. CTC determination might be improved beyond by the implementation of additional cytokeratins that are not covered by CellSearch® (e.g. CK7 and 20) [64].

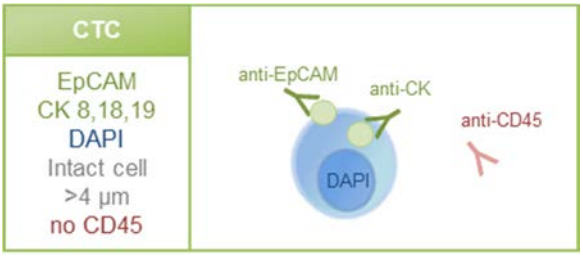

Figure 1-6: CellSearch® CTC definition. CTCs are defined as intact EpCAM-positive cells with at least 4 microns in size that stain positively for multiple CKs (predominantly CK8, 18, 19) and the nuclear dye DAPI, and lack staining for the leukocyte marker CD45.

1.3.2 CTC isolation and characterization

1.3.2.1 CTC enrichment

Since CTCs are rare events within the blood circulation and because specific tumor cell markers are missing, they are mainly isolated via a combination of enrichment and detection steps [65]. Many enrichment techniques have been developed and are currently under evaluation [66]. Generally, they follow two different strategies: selecting CTCs according to their physical properties (*marker-independent* approaches) or based on their biological features (*marker-dependent* approaches) [67] (**Table 1-1**).

Marker-independent CTC enrichment based on physical criteria employs various filtration technologies (e.g. ISET®, Parsortix, ScreenCell®) or Ficoll density-gradient approaches

[1] CellSearch® uses two monoclonal antibodies targeting CK4, 5, 6, 8, 10, 13, 18 (clone C11) and CK19 (clone A53.B/A2), whereas CK8, 18 and 19 are predominantly recognized [63, 64].

(e.g. OncoQuick™), which exploit the differences in size and deformability of CTCs in comparison to other cells found in the peripheral blood [68-70]. Like filter devices, label-free micro-fluidic approaches are promising tools that also allow CTC isolation without extensive pre-treatment of the blood (e.g. JETTA™, DFF-chip) [71, 72].

Table 1-1: Technologies for CTC enrichment and identification.

Technology	CTC enrichment (target)	CTC characterization	References
Marker-independent			
Ficoll/density gradient	Density centrifugation/negative depletion	ICC, PCR, FISH	[73, 74]
EPISPOT	Density gradient centrifugation or negative depletion	Immunological detection of secreted proteins (e.g. MUC1, CK19)	[75]
VitaAssay™	Ingestion of fluorescently-labeled matrix	ICC, PCR	[76]
OncoQuick™	Density gradient centrifugation	ICC, PCR	[68]
ISET®	Size (>8 µm)	ICC, PCR, FISH	[69]
Parsortix	Size (>10 µm)	ICC, PCR, FISH	ANGLE plc, UK
ScreenCell®	Size	ICC, PCR	[70, 77]
JETTA™	Size	ICC, PCR, FISH	[71]
DFF-chip	Size	ICC, PCR, FISH	[72]
Marker-dependent			
CellSearch® CTC/Profile Kit	EpCAM-ferrofluid	ICC for CK, CD45, DAPI; PCR, FISH	[78, 79]
IsoFlux®	EpCAM/antibody-coated beads	ICC, PCR, FISH	[80]
CTC/Herringbone-Chip	EpCAM/EpCAM, HER2, EGFR coated microposts	ICC for CK, CD45, DAPI/ EpCAM, CK5,7,8,18,19, CDH1, CDH2, PAI1, FN1; PCR	[34, 81]
Ariol® system	EpCAM/CK-coated microbeads	ICC for CK8, 18, 19, CD45, DAPI	[82]
GILUPI CellCollector™	EpCAM-coated wire	ICC	[83]
AdnaTest® Breast Cancer/EMT/stem cell	EpCAM, MUC1 ferrofluid	Multiplex RT-PCR for MUC1, GA733-2, HER2/TWIST, Akt2, PI3K, ALDH1	[84, 85]
Liquid bead array	Density gradient centrifugation/ EpCAM-ferrofluid	Multiplex PCR for CK19, HER2, MAGE-A3, hMAM, PBGD, TWIST1	[86]
Marker-independent and marker-dependent			
CTC-iChip	Size and EpCAM-based selection or negative depletion	ICC, PCR, FISH	[87]

ICC= immunocytochemistry; FISH= fluorescence in situ hybridization; CDH1/2= cadherin-1/2 (E-/N-cadherin); ALDH= aldehyde dehydrogenase; MAGE-A3= melanoma-associated antigen 3; PAI1= plasminogen activator inhibitor-1; FN1= fibronectin 1; MUC1= mucin-1; PBGD= porphobilinogen deaminase

Marker-dependent approaches, which are preferably used in the clinical and experimental setting, primarily use antibodies against EpCAM (e.g. CellSearch®, CTC/HB-Chip, IsoFlux®, AdnaTest®) [78, 80, 81, 84]. Among EpCAM-based strategies, the CellSearch®

assay (Janssen Diagnostics, LLC, USA) is currently representing the “gold standard” for CTC detection [63]. With this approach CTCs are isolated from 7.5 ml of peripheral blood using a ferrofluid which is coated with EpCAM-specific antibodies. Captured CTCs are then identified by the aforementioned criteria (**Figure 1-6**). Other techniques such as IsoFlux®, AdnaTest® or MACS also use EpCAM antibody-coated (micro-)beads for immunocapture of CTCs [80, 84]. In the microfluid channels of the CTC-/Herringbone-Chip, rare cells can be enriched from the whole blood with EpCAM antibody-coated microposts [34, 81]. Promising technologies enabling isolation of viable CTCs from larger blood volumes include leukapheresis [88], flow chambers, and the GILUPI CellCollector™ [83].
Since EpCAM is broadly used for CTC enrichment, the following section is dedicated to a more comprehensive description of EpCAM.

The Epithelial Cell Adhesion Molecule

The tumor-associated expression of EpCAM (CD326) was originally discovered more than 35 years ago in colon cancer [89, 90]. Thereafter, it was described independently by many research groups leading to a plethora of different synonyms and respective monoclonal antibodies targeting it (e.g. 17-1A, HEA125, VU1D9, TROP-1, ESA, KSA, GA733-2) [90].

EpCAM is a glycosylated transmembrane (type I) 39-42 kDa protein functioning as epithelial-specific cell adhesion molecule (CAM) that interferes with cadherin-mediated cell-cell contact [91, 92]. It is assembled by 314 amino acids (aa), from which 265 aa constitute the large extracellular domain with epidermal growth factor (EGF)- and thyroglobulin repeat-like domains, 23 aa make up the transmembrane domain and only 26 aa are facing the cytoplasm [90, 93, 94]. Although the function of EpCAM is not fully understood yet, recent data indicate that EpCAM bears a dual role: besides its function in homotypic cell adhesion, it represents a receptor being involved in cellular signaling processes (i.e. proliferation, migration, differentiation). Nevertheless, its structure does not resemble any of the classical CAMs including cadherins, selectins, integrins and the Ig-CAM superfamily [95, 96].

Initially, EpCAM activation is triggered by cell-to-cell contact [96]. More detailed, Maetzel *et al.* postulated that EpCAM is activated as a signal transducer via regulated intra-membrane proteolysis (by TACE, PS-2) resulting in the shedding of the extracellular domain (EpEX) and the release of its intracellular domain (EpICD) into the cytoplasm [96] (**Figure 1-7**). EpICD then becomes part of a signaling complex including *wnt* pathway components such as FHL2, β-catenin, and Lef-1, and translocates into the nucleus, whereupon target genes such as *c-myc*, *cyclins* and other genes involved in cell growth, proliferation and cell death are regulated [97, 98] . EpEX, in turn, was reported to act as a soluble ligand/agonist for

EpCAM-expressing cells and is thereby suggested to promote the generation of EpICD via a positive-feedback loop in an auto-/paracrine fashion [96].

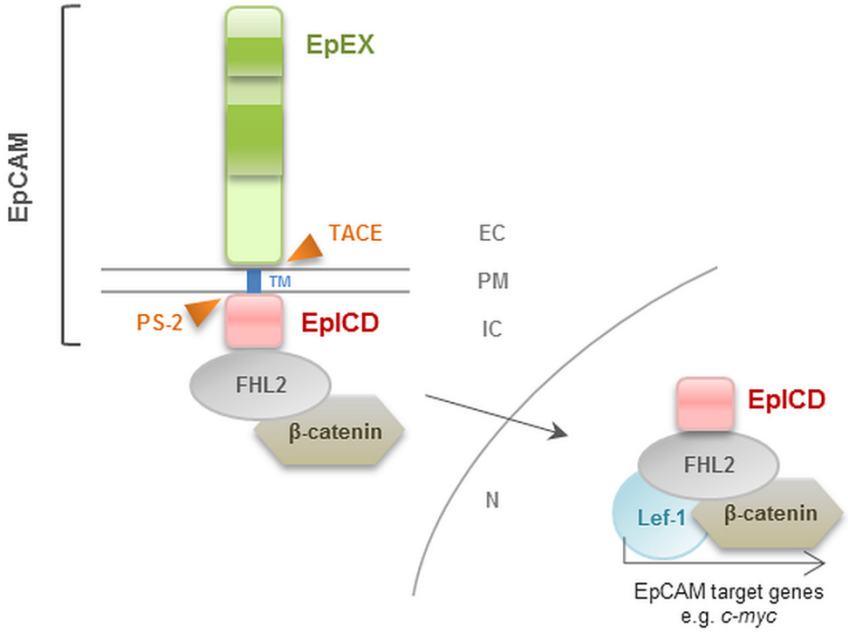

Figure 1-7: Schematic of EpCAM and its nuclear signaling. EpCAM is comprised of an extracellular domain (EpEX), a single transmembrane domain (TM) and an intracellular domain (EpICD). Upon intramembrane cleavage by TACE and presenilin-2 (PS-2) (indicated by ►) EpCAM gets activated. In a complex with FHL2 and β-catenin EpICD translocates into the nucleus (N). Within the nucleus this multiprotein complex interacts with Lef-1 and binds to DNA at Lef-1 consensus sites leading to the transcription of EpCAM-dependent genes (e.g. *c-myc*). (EC= extracellular, PM= plasma membrane, IC= intracellular). **Illustration and description according to [96].**

In humans, EpCAM is generally expressed in epithelia and neoplasias derived from epithelial tissues [91]. Within normal tissue EpCAM expression is found at the basolateral cell membrane located in intercellular spaces; in cancerous tissue it is more homogeneously distributed on the cell surface, making it more accessible to antibodies [94, 95]. Comparing normal and malignant tissue, EpCAM expression levels are usually enhanced in human carcinomas as it is for breast cancer [91]. Osta *et al.* found that EpCAM is overexpressed 100- to 1,000-fold in primary and metastatic breast cancer and siRNA silencing of EpCAM led to a decrease in cell proliferation, migration and invasion in different breast cancer cell lines. Furthermore, they showed that EpCAM is an antagonist to E-cadherin, negatively modulating cadherin-based cell adhesion. Thus, it is hypothesized that overexpression of EpCAM might be an important mechanism to disrupt cell-cell contacts and thereby mediating migration of cancer cells [95]. As a consequence, many therapeutic strategies were launched specifically targeting EpCAM [90] and EpCAM became one of the most commonly used capturing antigens for CTC isolation [66] (see chapter **1.3.2.1**). However, data indicate that EpCAM gets downregulated during dissemination, and further, that EMT

(chapter **1.2.2**) might be an important trigger behind this process [99, 100]. Punnoose *et al.* demonstrated that cancer cells with a more aggressive mesenchymal phenotype could escape EpCAM-based enrichment and additionally, Gorges *et al.* showed that EMT-traits were upregulated in EpCAM-negative tumor cells in breast cancer [101, 102]. Underlying molecular mechanisms that contribute to EpCAM modulation have been proposed by different groups: Gires *et al.* described that TNF-α might be responsible for EpCAM-downregulation [103] and Flieger *et al.* reported about EpCAM modulation via cytokines [104]. Another mechanism leading to the loss of EpCAM expression might be the hypermethylation of the *EPCAM* gene promoter [105]. Taken together, according to Gires and Stoecklein dynamic EpCAM expression can be either a consequence of EMT/MET or represents the active driving force within the regulation of the epithelial phenotype, whereby the latter role of EpCAM is currently favored [99]. As a consequence, EpCAM-dependent CTC enrichment strategies could lack in identifying the aggressive and/or stem-like CTCs, and alternative markers have to be determined that allow the efficient and specific enumeration of this $EpCAM^{low/neg}$ subpopulation. One strategy that deals with an EpCAM-independent CTC enrichment is the topic of the herein presented study using antibodies specific for different cell surface proteins (see **manuscript 3.1.2**).

1.3.2.2 CTC detection and characterization

Beyond tumor cell enrichment, detection and molecular characterization of CTCs is a crucial step towards gaining more insights into their mesenchymal, proliferative and stem cell-like features and the presence of effective therapeutic targets. CTCs can be detected and characterized using either molecular (DNA/RNA level), immunocytological (antibody-based, protein level) or functional assays [106].

DNA- and RNA-based assays

Analyses on the genomic level include, for example, the visualization of specific chromosomal regions or rearrangements using fluorescence *in situ* hybridization (FISH) [73]. Campos *et al.* extended this approach by combining it with immunofluorescence for protein expression (i.e. simultaneous CK expression and chromosome 17/HER2 gene status, FICTION) [107]. The so-called droplet digital PCR represents a technique enabling absolute nucleic-acid quantitation by a conventional TaqMan assay in a 96-well arrayed format. Using this method, detection of mutant DNA, quantitation of circulating DNA and germline copy number variations could be achieved [108, 109]. Recent advancements are mainly directed towards molecular characterization of single cells: whole genome amplification (WGA) allows the increase of small DNA amounts for subsequent analysis of

copy number alterations by array comparative genomic hybridization (aCGH) or point mutations by conventional and next-generation sequencing approaches [110, 111]. Templates for high-throughput sequencing might be also provided by BEAMing (=beads, emulsion, amplification, magnetics) PCR, wherein single DNA molecules are bound to streptavidin-coated beads and PCR reaction takes place in water-oil emulsions [112]. DNA-based molecular characterization of CTCs can be further realized by analysis of promoter methylation which is known to lead to epigenetic silencing of tumor suppressors. For instance, Chimonidou *et al.* reported about promoter methylation of CST6, BRMS1 and SOX17 in EpCAM-positive CTCs from patients with operable and metastatic breast cancer [113].

RNA-based approaches detect CTCs upon capture of epithelial mRNA. Commonly used markers include CKs, EpCAM and mammaglobin, whereas detection or quantification of mRNA encoding CK19 has been the most widely applied method in clinical trials [114-116]. The commercially available AdnaTest® utilizes non-quantitative RT-PCR to identify putative transcripts of genes after immunomagnetic separation of EpCAM/MUC1-positive CTCs [117]. Alternatively, microarray analysis of metastatic breast cancer samples conducted by LeBleu *et al.* detected PGC-1 α gene expression in a high percentage of CTCs undergoing EMT. PGC-1 α induces mitochondrial biogenesis and oxidative phos-phorylation to promote ATP production during bioenergetic crisis and thereby might foster cancer invasion and metastasis [118]. Single CTC profiling on transcriptome level was first described by Powell *et al.* who included 87 cancer-associated and reference genes in their microfluidic chip-based multiplexed qRT-PCR assay. Elevated transcript levels of metastasis (e.g. S100A4) and EMT (e.g. vimentin, ZEB2, TGF-β1) genes in CTCs revealed transcriptional heterogeneity and diversity from cell lines. Furthermore, they found that individual CTCs did not cluster by patient or disease stage [119]. More recently, single CTC mRNA-sequencing studies in pancreatic and breast cancer allowed to investigate differences in global mRNA signatures within matched patient samples (e.g. CTCs, CTC clusters, leukocytes) [120, 121]. Additionally, the RNA/CTCscope assay, which detects CTCs via a multiplex RNA *in situ* hybridization (ISH), represents a quite promising tool to visualize RNA molecules in single CTCs [122, 123]. Interestingly, applying RNA ISH of pooled epithelial and mesenchymal transcripts uncovered an association between expression of mesenchymal markers and CTC cluster formation [54]. Gasch *et al.* further reported about an ISH protocol, incorporating the CellSearch® CTC detection system, which enables the clinical investigation of micro-RNAs in single CTCs. Using this method, they could demonstrate heterogeneity of miR-10b expression in individual breast cancer CTCs [124].

Immunocytological assays

Antibody-based detection approaches such as immunofluorescence and immunocytochemistry enable the identification of CTCs via distinct staining patterns as well as specific morphological features distinguishing cancer cells from normal blood cells [125, 126]. Standard markers for CTC – and also DTC (disseminated tumor cell) - detection in breast cancer are antibodies against cytoskeletal (CKs 8/18/19) and epithelial surface proteins (e.g. E-cadherin, EpCAM) [54, 78, 84, 127]. In the case of decreased expression of proteins normally associated with epithelial cells (e.g. during EMT, as discussed above) additional mesenchymal markers (e.g. AKT2, PI3K, SNAI1, SNAI2, TWIST1, vimentin, N-cadherin) can be employed for detection/characterization of mesenchymal-like CTCs [46, 85, 128-132]. Regarding metastatic breast cancer, in a cohort of 19 patients TWIST1 and SNAI1 could be detected in 26.3 and 21.1% of the samples, respectively [133]. In a study of Aktas *et al.* TWIST1, AKT2 and PI3K were identified in 42, 62 and 58% of 26 CTC-positive samples from 39 patients [134]. Alternatively, tumor-associated markers such as HER2 and EGFR [54, 84] or tissue-specific markers including mammaglobin [86] can be employed for CTC characterization in breast cancer samples. The CTCs' ability to enter dormancy has been linked to stem cell-like features enclosing self-renewal capabilities. Consequently, "stemness" markers (e.g. ALDH1 (aldehyde dehydrogenase), CD133, CD44) might underpin CTC isolation and subsequent characterization [74, 133-135]. Such stem-cell like CTCs could be identified e.g. by Theodoropoulos *et al.* who showed that patients with metastatic breast cancer harbor CTCs with a $CD44^{pos}/CD24^{neg/low}$ and $ALDH1^{high}/CD24^{neg/low}$ phenotype, respectively [74]. Giordano *et al.* further observed that patients with EMT-like CTCs were increased in the $ALDH^{pos}/CD133^{pos}$ cancer cell fraction [135].

Functional assays

To unravel the biology of CTCs and to uncover cells that are responsible for initiating metastasis, it is of utmost importance to perform functional assays. Efforts in this direction have been undertaken both *in vitro* and *in vivo. In vitro* assays detect viable CTCs, either by specific protein secretion generating "immunospots" visualized by fluorescently-labeled antibodies (EPISPOT) [75] or via engulfment of a fluorescently-labeled collagen adhesion matrix (VitaAssay™) by CTCs [76]. Further *in vitro* approaches include the direct expansion/culture of human CTCs by applying different media with varying growth factor supplements or specific culture conditions, e.g. under hypoxia [136-138]. According to Yu *et al.* CTCs from patients with metastatic disease could be kept in culture for more than 6 months under hypoxic conditions [137]. Cayrefourcq *et al.* were able to establish the first *ex*

vivo colon CTC line, which was stable for at least 16 months and showed important features of the colon cancer in the patient [138]. Alternatively, expansion of CTCs can be achieved *in vivo* by xenotransplantation models. Baccelli *et al*. transplanted CTCs from 110 breast cancer patients with progressive disease into immunodeficient mice. After transplantation, only recipient mice receiving more than 1,000 CTCs from three different luminal BC patients developed blood-borne metastases, indicating that these patients harbor functional metastasis-initiating cells (MICs). Furthermore, they showed that the MIC-containing CTC population were among a cell population with $EpCAM^{low}/CD44^{high}/ CD47^{high}/c\text{-}Met^{high}$ phenotype [139]. More recently, Hodgkinson *et al.* demonstrated that CTCs from patients with small-cell lung cancer are tumorigenic upon injection into immunocompromised mice and reflect tumor biology and *ex vivo* treatment response [140].

1.3.3 Clinical role of CTCs

The potential of CTCs for clinical applications can lie in different stages of the disease including early cancer prevention, identification of minimal residual disease, monitoring of therapy and the identification of predictive biomarkers/druggable targets [29]. Furthermore, CTCs have been confirmed as prognostic markers in many clinical studies for both, primary [141-143] and metastatic [127, 132, 144-146] breast cancer. While CTCs' role in disease prevention is not defined yet, their great potential could be shown for therapy monitoring, especially in case of metastatic breast cancer (**Table 1-2**). The known strength of CTCs for clinical applications promoted the inclusion of the CTC status into a new edition of the TNM (tumor, node, metastasis) cancer staging manual: $cM_0(i+)$, i.e. no clinical signs of metastasis, but isolated tumor cells in either blood, lymph nodes or bone marrow are detected. However, since the clinical utility of CTCs has not been fully clarified, the inclusion into the ASCO (American Society of Clinical Oncology) or other clinical guidelines has yet to be realized [106].

Table 1-2: Clinical applications of CTCs in metastatic and early breast cancer.

CTCs as tool for...	Metastatic breast cancer	Early breast cancer
Prognosis	≥5 CTCs in 7.5 ml blood correlate with shorter PFS and OS [127]	CTC detection correlates significantly with DFS and OS (SUCCESS trial [141])
Therapy monitoring	High CTC levels after start of first-line chemotherapy can predict progression, however, patients do not benefit from a regimen switch (clinical trials: SWOG 0500 [147], ongoing: CirCe01 [148])	No clinical consequence so far
Treatment decision	Evidence pending (ongoing clinical trials: STIC CTC METABREAST, DETECT III/IVa/IVb/V [148])	Evidence pending (ongoing clinical trials: TREAT CTC [148])

PFS= progression-free survival; OS= overall survival; DFS= disease-free survival

1.3.3.1 CTCs as prognostic tool

Early diagnosis is crucial for successful treatment and good prognosis in breast cancer. But still, CTCs can be detected in approx. 10-60% of newly diagnosed non-metastatic patients [149-151]. These CTCs may persist beyond primary treatment and are considered to be the founders of metastasis. Consequently, 20-30% of patients suffer from metastatic relapse after several months or years after early diagnosis [152].

Metastatic breast cancer

Approximately 40-80% of metastatic breast cancer patients harbor CTCs in their peripheral blood [153]. Already more than ten years ago Cristofanilli *et al*. delivered first impressive hints that metastatic breast cancer patients with at least 5 CTCs per 7.5 ml blood have both significantly shorter progression-free survival (PFS) and overall survival (OS) compared to patients with less than 5 CTCs (CellSearch® analysis) [127]. Upon these findings a cut-off of ≥5 CTCs/7.5 ml blood was set in order to distinguish patients with favorable from those with poor clinical outcome. This strong prognostic significance of CTCs regarding PFS and OS could be also confirmed by a recent pooled analysis of 20 studies throughout Europe [146]. Furthermore, CTCs' use as prognostic tool concerning different molecular subtypes of primary tumors was evaluated in several retrospective and prospective studies [132, 144, 145]. For example, Wallwiener *et al.* reported that the prognostic significance of CTCs in metastatic breast cancer patients is independent of the molecular subtype.

Early breast cancer

In early breast cancer, CTC presence has also been linked to prognosis and thereby seems to be independent of tumor grade, histopathology, nodal status or Ki67 expression [142, 154]. However, mixed associations were found for different breast cancer subtypes regarding early and metastatic breast cancer. While Boutrus *et al.* reported that CTCs are prognostic in early breast cancer patients with $ER^{neg}PR^{neg}HER2^{neg}$ or $ER^{neg}PR^{neg}HER2^{pos}$, but not in ER^{pos} tumors [155], Giordano *et al*. showed prognostic significance of CTCs in all breast cancer subtypes except for $HER2^{pos}$ tumors [132]. Besides, a meta-analysis conducted by Zhang *et al.* confirmed CTCs as stable prognosticators regarding OS and disease-free survival (DFS) in early breast cancer [143]. Furthermore, strong evidence has also been provided by the German SUCCESS trial, wherein patients before and after chemotherapy were included. After surgery/before start of chemotherapy 21.5% of the patients were CTC-positive. After 3 years, clear prognostic relevance of CTCs concerning DFS, distant disease-free survival (DDFS) and OS was demonstrated [141].

1.3.3.2 CTCs as therapy monitoring tool

Since the half-life of CTCs within the blood circulation is considered to be in the range of minutes, they may represent an attractive and reliable “liquid biopsy” tool to monitor therapy efficacy and disease progression instead of conventional imaging technologies [156, 157].

Metastatic breast cancer

Regarding metastatic breast cancer, changes in CTC numbers during the course of treatment were shown to correlate with clinical and radiological response [147]. In a study from Hayes *et al.* patients with initially high CTC levels showed decreased CTC counts after start of first-line chemotherapy with a significantly longer PFS/OS and identical prognosis to CTC-negative patients [158]. Nakamura *et al.* observed in their prospective multi-center study that the change in CTC numbers highly correlated with results from imaging before and after therapy assuming that CTCs can be a biomarker allowing earlier prediction of treatment efficacy than imaging modalities [159]. In a further study conducted by Hartkopf *et al.* CTC levels of 58 patients at the beginning and after 3 cycles of chemotherapy were determined and correlated to radiologic ‘Response Evaluation Criteria In Solid Tumors’ (RECIST) criteria. Therein, changing CTC levels significantly correlated with response to therapy and were associated with significantly increased OS in patients with decreasing CTC numbers [160]. Taking up on these findings, the SWOG S0500 trial addressed whether a treatment switch leads to a CTC decrease in patients that initially did not respond to therapy. Interestingly, alternate cytotoxic therapy did not significantly influence CTC

numbers or had an impact on OS compared to standard chemotherapy in these patients [147]. The similar, still ongoing CirCe01 trial evaluates CTC counts serially after each treatment cycle, with patients in the intervention arm switching regimen in case of adverse CTC levels [148]. Data have not been published so far.

Early breast cancer

Although there is evidence that some patients might benefit from an extended adjuvant therapy, the potential of CTC assessment in early breast cancer has not been clarified so far. The SUCCESS study is the first trial to provide strong evidence for the prognostic relevance of CTCs in early breast cancer before and after adjuvant chemotherapy in a large patient cohort. Therein, CTC persistence during adjuvant chemotherapy was associated with reduced OS [141]. Furthermore, within a subarm of the Neo-ALTTO study, which includes patients with detectable CTCs at baseline and/or 2/18 weeks after start of chemotherapy, significantly less pathological complete remission (27% *vs* 42%) was described [161]. In a further study reported by Xenidis *et al*. a significant improvement of the DFS could be achieved in patients receiving a taxane-based chemotherapy accompanied by a higher incidence of CTC elimination compared to the taxane-free treated group [162].

1.3.3.3 CTCs as therapy decision-making tool

Several clinical trials are currently underway in order to test CTCs' utility as therapy selection tool for both metastatic and early breast cancer. It is known that the protein expression profiles of CTCs might differ from those of the corresponding primary tumors [149, 163-165]. HER2 has been the most extensively addressed marker and since treatment decisions are mainly made based on primary tumor characteristics, targeting CTCs might be decisive with respect to personalized therapy selection entailing benefits for the patients.

Metastatic breast cancer

As mentioned above (chapter **1.1.2.3**) metastatic breast cancer can be regarded as a heterogeneous malignancy with dynamic phenotypic changes that may occur over time. Consequently, predictive markers (e.g. HER2, ER, and PR) can be affected by these changes. Therapeutic target evaluation on CTCs as a substitute for biopsy of the metastases therefore might be helpful in gaining more insights into potential drug resistance mechanisms of the metastases. Considering HER2 discrepancy, the ongoing prospective multi-center DETECT III study is randomizing patients with $HER2^{neg}$ tumors and $HER2^{pos}$

CTCs to standard therapy with or without HER2-targeted therapy with lapatinib, a tyrosine kinase inhibitor [148]. In continuation, the DETECT IVa/b trial will analyze the effectiveness of the mTOR-inhibitor everolimus in combination with endocrine agents/eribulin on CTCs in patients with $HER2^{neg}$ CTCs and $HER2^{neg}/HR^{pos}$ primary tumors. Since persistent high CTC levels might require more aggressive treatment options, the STIC CTC METABREAST trial randomizes HR^{pos} patients between the clinician choice and CTC count-driven choice. Patients within the CTC-arm with ≥5 CTCs/7.5 ml will receive chemotherapy, whereas patients with lower CTC levels will receive endocrine therapy only [148]. As even high discordance rates with respect to ER (and PR) expression were reported for metastatic breast cancer patients [165], there are also implications for treatment of ER^{pos} CTCs in patients with ER^{neg} primary tumors. However, it still remains unclear if patients benefit from systemic therapies selected upon CTC expression profiles [166].

Early breast cancer

In case of early breast cancer the phase II Treat-CTC trial addresses whether the eradication of CTCs correlates with a better outcome by randomizing $HER2^{neg}$ patients with detectable $HER2^{pos}$ CTCs to HER2-therapy with trastuzumab (monoclonal antibody against HER2) *versus* observation [148]. Besides, HER2-targeted treatment was also able to clear HER2-positive DTCs in the bone marrow in recurrence-free patients as reported by Rack *et al.* [167]. In a study with 431 patients Fehm *et al.* found that the assessed CTCs displayed a rather triple-negative ($ER^{neg}PR^{neg}HER2^{neg}$) phenotype regardless of the primary tumor characteristics. Considering this loss of ER-positivity in CTCs, they state that the impact on adjuvant treatment can only be answered in clinical trials which randomize patients according to the expression profiles of their CTCs [84].

1.3.4 Challenges in CTC research

Over the past years CTCs gained tremendous attention: as putative founders of metastatic disease, their molecular analysis may provide important insights into mechanisms of tumorigenesis, geno- and phenotypic intra-/interpatient variability and therapeutic treatment options. Although their clinical value as potential early cancer detection, diagnostic, prognostic, predictive and surrogate biomarkers has been extensively studied and partly demonstrated, the obvious strength of CTC-based opportunities is closely interrelated to drawbacks and challenges that one has to face in the CTC field (**Figure 1-8**). Consequently, jumping to conclusions should be avoided [106, 168].

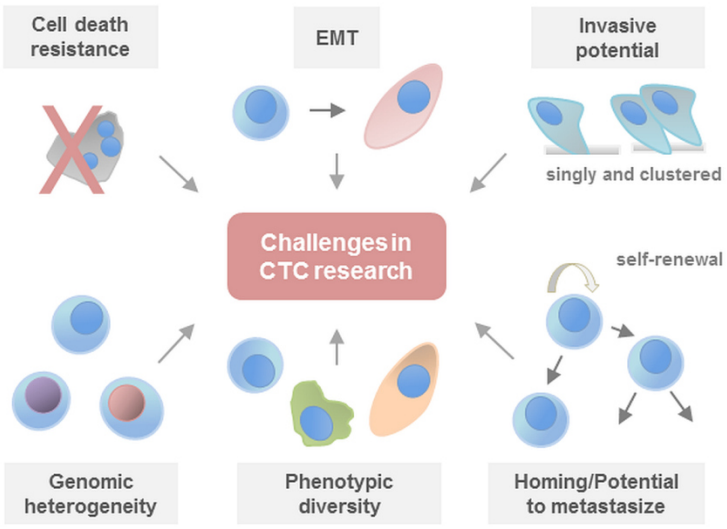

Figure 1-8: CTC characteristics as challenges in CTC research. Further explanations are given in the main text. **Modified after [106].**

The low frequency of CTCs can be regarded as one of the major challenges in CTC research. Moreover, due to their genomic heterogeneity (e.g. *ER*, *HER2*, *PIK3CA*) and phenotypic diversity (e.g. morphology, size) CTCs obtained by blood withdrawal might not fully reflect the entire cell population present in the blood or in the tumor they originated from. Additionally, blood sample preparation should ensure only minimal cell loss. Sample size as a limiting factor can be overcome by collecting CTCs directly via the GILUPI CellCollector® [83] or increasing the blood volume by leukapheresis [88]. Methodological constraints are further linked to interoperator variability and to subjectivity in interpretation of the results, respectively.

CTC enrichment is mainly based on the epithelial marker EpCAM. However, EMT leads to cell plasticity and intermediate or even "organ-mimetic" phenotypes, respectively, which demands for alternative, highly specific/sensitive capture and detection strategies to eliminate false-positive and false-negative findings (see **manuscript 3.1.2**). Simultaneously, these tumor cell phenotypes might be accompanied by long term persistence and the acquisition of stem-cell like features such as self-renewal/quiescence or an $EpCAM^{low/neg}/CD44^{pos}/CD24^{neg}/ALDH^{pos}$ phenotype resulting in resistance to cell death and to chemotherapy. The exact mechanisms, by which viable CTCs are able to obtain aggressive or metastasis-initiating potential, still have to be elucidated. Likewise, the scientific/clinical value of homo- and heterotypic cell clusters merits further investigation.

1.4 References

1. Cancer Fact sheet N°297: World Health Organization; 2014 [updated Feb 2015]. Available from: http://www.who.int/mediacentre/factsheets/fs297/en/.
2. Weinberg RA. The Biology of Cancer. 2nd ed: *GS Garland Science*; 2013 May 15.
3. Pecorino L. Molecular biology of cancer. 2nd ed: *Oxford University Press*; 2008.
4. Stewart BW, Wild CP. World Cancer Report: International Agency for Research on Cancer, *WHO Press*; 2014.
5. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell*. 2011;144(5):646-74.
6. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JWW, Comber H, et al. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries in 2012. *Eur J Cancer*. 2013;49(6):1374-403.
7. DeSantis C, Ma J, Bryan L, Jemal A. Breast cancer statistics, 2013. *CA-Cancer J Clin*. 2014;64(1):52-62.
8. Tao Z, Shi A, Lu C, Song T, Zhang Z, Zhao J. Breast Cancer: Epidemiology and Etiology. *Cell Biochem Biophys*. 2015;72(2):333-8.
9. Krebsregisterdaten, ICD-10 C50, 2011: Zentrum für Krebsregisterdaten, Robert-Koch-Institut; 2015. Available from: http://www.krebsdaten.de/Krebs/DE/Content/Publikationen/Krebs_in_Deutschland/kid_2013/kid_2013_c50_brust.pdf?__blob=publicationFile.
10. Krebs in Deutschland 2009/2010. 9 ed: Robert Koch-Institut (Hrsg) und die Gesellschaft der epidemiologischen Krebsregister in Deutschland e.V. (Hrsg); 2013.
11. Goldberg J, Borgen P. Breast cancer susceptibility testing: past, present and future. *Expert Rev Anticancer Ther*. 2006;6(8):1205-14.
12. Antoniou AC, Cunningham AP, Peto J, Evans DG, Lalloo F, Narod SA, et al. The BOADICEA model of genetic susceptibility to breast and ovarian cancers: updates and extensions. *Br J Cancer*. 2008;98(8):1457-66.
13. Bertos NR, Park M. Breast cancer - one term, many entities? *J Clin Invest*. 2011;121(10):3789-96.
14. Available from: http://weill.cornell.edu/cms/health_library/images/ei_0192.gif.

15. Available from: http://www.breastcancer.org/Images/dcis_range_tcm8-78725.jpg.

16. Available from: https://www.krebsinformationsdienst.de/untersuchung/tnm.php.

17. Fumagalli D, Andre F, Piccart-Gebhart M, Sotiriou C, Desmedt C. Molecular biology in breast cancer: should molecular classifiers be assessed by conventional tools or by gene expression arrays? *Crit Rev Oncol Hematol*. 2012;84(Supplement 1):e58-69.

18. Polyak P. Heterogeneity in breast cancer. *J Clin Invest*. 2011;121(10):3786-8.

19. Higgins MJ, Baselga J. Targeted therapies for breast cancer. *J Clin Invest*. 2011;121(10):3797-803.

20. Holohan C, Van Schaeybroeck S, Longley D, Johnston P. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer*. 2013;13(10):714–26.

21. Eichhorn PJA, Gili M, Scaltriti M, Serra V, Guzman M, Nijkamp W, et al. Phosphatidylinositol 3-Kinase Hyperactivation Results in Lapatinib Resistance that Is Reversed by the mTOR/Phosphatidylinositol 3-Kinase Inhibitor NVP-BEZ235. *Cancer Res*. 2008;68(22):9221-30.

22. Kataoka Y, Mukohara T, Shimada H, Saijo N, Hirai M, Minami H. Association between gain-of-function mutations in PIK3CA and resistance to HER2-targeted agents in HER2-amplified breast cancer cell lines. *Ann Oncol*. 2010;21(2):255-62.

23. Jensen J, Laenkholm A-V, Knoop A, Ewertz M, Bandaru R, Liu W, et al. PIK3CA Mutations May Be Discordant between Primary and Corresponding Metastatic Disease in Breast Cancer. *Clin Cancer Res*. 2011;17(4):667-77.

24. Fehm T, Mueller V, Aktas B, Janni W, Schneeweiss A, Stickeler E, et al. HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. *Breast Cancer Res Treat*. 2010;124(2):403-12.

25. Hurst C, Zuiverloon T, Hafner C, Zwarthoff E, Knowles M. A SNaPshot assay for the rapid and simple detection of four common hotspot codon mutations in the PIK3CA gene. *BMC Res Notes*. 2009;2(1):66.

26. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer*. 2003;3(6):453-8.

27. DeVita VJ, Young R, Canellos G. Combination versus single agent chemotherapy: a review of the basis for selection of drug treatment of cancer. *Cancer*. 1975:98-110.

28. Gupta G, Massagué J. Cancer Metastasis: Building a Framework. *Cell*. 2006;127(4):679-95.

29. Pantel K, Speicher MR. The biology of circulating tumor cells. *Oncogene*. 2015:1-9.

30. Vanharanta S, Massagué J. Origins of Metastatic Traits. *Cancer Cell*. 2013;24(4):410-21.

31. Cancer Invasion and Metastasis: Molecular and Cellular Perspective. In: Martin T, Ye L, Sanders A, et al., editors.: Madame Curie Bioscience Database [Internet]. Austin (TX): *Landes Bioscience*; 2000-. 2013.

32. Deryugina EI, Quigley JP. Tumor angiogenesis: MMP-mediated induction of intravasation- and metastasis-sustaining neovasculature. *Matrix Biol*. 2015;44-46:94-112.

33. Robinson BD, Sica GL, Liu Y-F, Rohan TE, Gertler FB, Condeelis JS, et al. Tumor Microenvironment of Metastasis in Human Breast Carcinoma: A Potential Prognostic Marker Linked to Hematogenous Dissemination. *Clin Cancer Res*. 2009;15(7):2433-41.

34. Stott SL, Hsu C-H, Tsukrov DI, Yu M, Miyamoto DT, Waltman BA, et al. Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. *Proc Natl Acad Sci U S A*. 2010;107(43):18392-7.

35. Miles F, Pruitt F, van Golen K, Cooper C. Stepping out of the flow: capillary extravasation in cancer metastasis. *Clin Exp Metastas*. 2008;25(4):305-24.

36. Crissman JD, Hatfield JS, Menter DG, Sloane B, Honn KV. Morphological Study of the Interaction of Intravascular Tumor Cells with Endothelial Cells and Subendothelial Matrix. *Cancer Res*. 1988;48(14):4065-72.

37. Paget S. The distribution of secondary growths in cancer of the breast. *Lancet*. 1889;133(3421):571-3.

38. Nguyen DX, Bos PD, Massague J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer*. 2009;9(4):274-84.

39. Schmidt-Kittler O, Ragg T, Daskalakis A, Granzow M, Ahr A, Blankenstein TJF, et al. From latent disseminated cells to overt metastasis: Genetic analysis of systemic breast cancer progression. *Proc Natl Acad Sci U S A*. 2003;100(13):7737-42.

40. Hoffman P, Mauer A, Vokes E. Lung cancer. *Lancet*. 2000;355(9211):479-85.

41. Braun S, Vogl FD, Naume B, Janni W, Osborne MP, Coombes RC, et al. A Pooled Analysis of Bone Marrow Micrometastasis in Breast Cancer. *New Engl J Med*. 2005;353(8):793-802.

42. Klein CA. The Metastasis Cascade. *Science*. 2008;321(5897):1785-7.

43. Klein CA. Parallel progression of primary tumours and metastases. *Nat Rev Cancer*. 2009;9:302-12.

44. Nisticò P, Bissell M, Radisky D. Epithelial-Mesenchymal Transition: General Principles and Pathological Relevance with Special Emphasis on the Role of Matrix Metalloproteinases. *Cold Spring Harb Perspect Biol*. 2012;4:a011908.

45. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*. 2002;2(6):442-54.

46. Thiery JP, Acloque H, Huang RYJ, Nieto MA. Epithelial-Mesenchymal Transitions in Development and Disease. *Cell*. 2009;139(5):871-90.

47. Kalluri R, Weinberg R. The basics of epithelial-mesenchymal transition. *J Clin Invest*. 2009;119(6):1420-8.

48. Nieto MA. The Ins and Outs of the Epithelial to Mesenchymal Transition in Health and Disease. *Annu Rev Cell Dev Biol*. 2010;27:347-76.

49. Thiery J, Sleeman J. Complex networks orchestrate epithelial–mesenchymal transitions. *Nat Rev Mol Cell Biol*. 2006;7(2):131-42.

50. Acloque H, Adams M, Fishwick K, Bronner-Fraser M, Nieto M. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *J Clin Invest*. 2009;119(6):1438-49.

51. Maier HJ, Wirth T, Beug H. Epithelial-Mesenchymal Transition in Pancreatic Carcinoma. *Cancers*. 2010;2(4):2058-83.

52. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial–mesenchymal transition. *Nat Rev Mol Cell Biol*. 2014;15(3):178-96.

53. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell*. 2008;14(6):818-29.

54. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science*. 2013;339(6119):580-4.

55. Sarrió D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, Palacios J. Epithelial-Mesenchymal Transition in Breast Cancer Relates to the Basal-like Phenotype. *Cancer Res*. 2008;68(4):989-97.

56. Ashworth TR. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. *Med J*. 1869;14:146-9.

57. Joosse SA, Gorges TM, Pantel K. Biology, detection, and clinical implications of circulating tumor cells. *EMBO Mol Med*. 2014;7(1):1-11.

58. Butler TP, Gullino PM. Quantitation of Cell Shedding into Efferent Blood of Mammary Adenocarcinoma. *Cancer Res*. 1975;35(3):512-6.

59. Meng S, Tripathy D, Frenkel EP, Shete S, Naftalis EZ, Huth JF, et al. Circulating Tumor Cells in Patients with Breast Cancer Dormancy. *Clin Cancer Res*. 2004;10(24):8152-62.

60. Coumans FAW, van Dalum G, Beck M, Terstappen LWMM. Filter Characteristics Influencing Circulating Tumor Cell Enrichment from Whole Blood. *PLoS ONE*. 2013;8(4):e61770.

61. Galanzha EI, Zharov VP. Circulating Tumor Cell Detection and Capture by Photoacoustic Flow Cytometry in Vivo and ex Vivo. *Cancers*. 2013;5(4):1691-738.

62. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor Cells Circulate in the Peripheral Blood of All Major Carcinomas but not in Healthy Subjects or Patients With Nonmalignant Diseases. *Clin Cancer Res*. 2004;10(20):6897-904.

63. Riethdorf S, Fritsche H, Müller V, Rau T, Schindlbeck C, Rack B, et al. Detection of Circulating Tumor Cells in Peripheral Blood of Patients with Metastatic Breast Cancer: A Validation Study of the CellSearch System. *Clin Cancer Res*. 2007;13(3):920-8.

64. de Wit S, van Dalum G, Lenferink A, Tibbe A, Hiltermann T, Groen H, et al. The detection of EpCAM(+) and EpCAM(-) circulating tumor cells. *Sci Rep*. 2015;5:12270.

65. Mostert B, Sleijfer S, Foekens JA, Gratama JW. Circulating tumor cells (CTCs): Detection methods and their clinical relevance in breast cancer. *Cancer Treat Rev*. 2009;35:463-74.

66. Barradas AMC, Terstappen LWMM. Towards the Biological Understanding of CTC: Capture Technologies, Definitions and Potential to Create Metastasis. *Cancers*. 2013;5(4):1619-42.

67. Broersen LA, van Pelt G, Tollenaar REM, Mesker W. Clinical application of circulating tumor cells in breast cancer. *Cell Oncol*. 2014;37(1):9-15.

68. Müller V, Stahmann N, Riethdorf S, Rau T, Zabel T, Goetz A, et al. Circulating Tumor Cells in Breast Cancer: Correlation to Bone Marrow Micrometastases, Heterogeneous Response to Systemic Therapy and Low Proliferative Activity. *Clin Cancer Res*. 2005;11(10):3678-85.

69. Lin HK, Zheng S, Williams AJ, Balic M, Groshen S, Scher HI, et al. Portable Filter-Based Microdevice for Detection and Characterization of Circulating Tumor Cells. *Clin Cancer Res*. 2010;16(20):5011-8.

70. Freidin MB, Tay A, Freydina DV, Chudasama D, Nicholson AG, Rice A, et al. An assessment of diagnostic performance of a filter-based antibody-independent peripheral blood circulating tumour cell capture paired with cytomorphologic criteria for the diagnosis of cancer. *Lung Cancer*. 2014;85(2):182-5.

71. Riahi R, Gogoi P, Sepehri S, Zhou YI, Handique K, Godsey JIM, et al. A novel microchannel-based device to capture and analyze circulating tumor cells (CTCs) of breast cancer. *Int J Oncol*. 2014;44(6):1870-8.

72. Hou HW, Warkiani ME, Khoo BL, Li ZR, Soo RA, Tan DS-W, et al. Isolation and retrieval of circulating tumor cells using centrifugal forces. *Sci Rep*. 2013;3:1259.

73. Fehm T, Sagalowsky A, Clifford E, Beitsch P, Saboorian H, Euhus D, et al. Cytogenetic Evidence That Circulating Epithelial Cells in Patients with Carcinoma Are Malignant. *Clin Cancer Res*. 2002;8(7):2073-84.

74. Theodoropoulos PA, Polioudaki H, Agelaki S, Kallergi G, Saridaki Z, Mavroudis D, et al. Circulating tumor cells with a putative stem cell phenotype in peripheral blood of patients with breast cancer. *Cancer Lett*. 2010;288(1):99-106.

75. Alix-Panabières C. EPISPOT Assay: Detection of Viable DTCs/CTCs in Solid Tumor Patients. In: Ignatiadis M, Sotiriou C, Pantel K, editors. Minimal Residual Disease and Circulating Tumor Cells in Breast Cancer. Recent Results in Cancer Research. 195: Springer Berlin Heidelberg; 2012. p. 69-76.

76. Lu J, Fan T, Zhao Q, Zeng W, Zaslavsky E, Chen JJ, et al. Isolation of circulating epithelial and tumor progenitor cells with an invasive phenotype from breast cancer patients. *Int J Cancer*. 2010;126(3):669-83.

77. Kulemann B, Pitman MB, Liss AS, Valsangkar N, Fernández-del Castillo C, Lillemoe KD, et al. Circulating Tumor Cells Found in Patients With Localized and Advanced Pancreatic Cancer. *Pancreas*. 2015;44(4):547-50.

78. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, et al. Circulating Tumor Cells: A Novel Prognostic Factor for Newly Diagnosed Metastatic Breast Cancer. *J Clin Oncol*. 2005;23(7):1420-30.

79. Sieuwerts A, Kraan J, Bolt-de Vries J, Spoel P, Mostert B, Martens JM, et al. Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. *Breast Cancer Res Treat*. 2009;118(3):455-68.

80. Harb W, Fan A, Tran T, Danila DC, Keys D, Schwartz M, et al. Mutational Analysis of Circulating Tumor Cells Using a Novel Microfluidic Collection Device and qPCR Assay. *Transl Oncol*. 2013;6(5):528-38.

81. Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature*. 2007;450(7173):1235-9.

82. Deng G, Herrler M, Burgess D, Manna E, Krag D, Burke JF. Enrichment with anti-cytokeratin alone or combined with anti-EpCAM antibodies significantly increases the sensitivity for circulating tumor cell detection in metastatic breast cancer patients. *Breast Cancer Res*. 2008;10(4):R69.

83. Saucedo-Zeni N, Mewes S, Niestroj R, Gasiorowski L, Murawa D, Nowaczyk P, et al. A novel method for the in vivo isolation of circulating tumor cells from peripheral blood of cancer patients using a functionalized and structured medical wire. *Int J Oncol*. 2012;41(4):1241-50.

84. Fehm T, Hoffmann O, Aktas B, Becker S, Solomayer EF, Wallwiener D, et al. Detection and characterization of circulating tumor cells in blood of primary breast cancer patients by RT-PCR and comparison to status of bone marrow disseminated cells. *Breast Cancer Res*. 2009;11(4):R59.

85. Kasimir-Bauer S, Hoffmann O, Wallwiener D, Kimmig R, Fehm T. Expression of stem cell and epithelial-mesenchymal transition markers in primary breast cancer patients with circulating tumor cells. *Breast Cancer Res*. 2012;14(1):R15.

86. Markou A, Strati A, Malamos N, Georgoulias V, Lianidou ES. Molecular Characterization of Circulating Tumor Cells in Breast Cancer by a Liquid Bead Array Hybridization Assay. *Clin Chem*. 2011;57(3):421-30.

87. Ozkumur E, Shah AM, Ciciliano JC, Emmink BL, Miyamoto DT, Brachtel E, et al. Inertial Focusing for Tumor Antigen–Dependent and –Independent Sorting of Rare Circulating Tumor Cells. *Sci Transl Med*. 2013;5(179):179ra47.

88. Fischer JC, Niederacher D, Topp SA, Honisch E, Schumacher S, Schmitz N, et al. Diagnostic leukapheresis enables reliable detection of circulating tumor cells of nonmetastatic cancer patients. *Proc Natl Acad Sci U S A*. 2013;110(41):16580-5.

89. Herlyn D, Herlyn M, Steplewski Z, Koprowski H. Monoclonal antibodies in cell-mediated cytotoxicity against human melanoma and colorectal carcinoma. *Eur J Immunol*. 1979;9(8).

90. Baeuerle PA, Gires O. EpCAM (CD326) finding its role in cancer. *Br J Cancer*. 2007;96(3):417-23.

91. Patriarca C, Macchi RM, Marschner AK, Mellstedt H. Epithelial cell adhesion molecule expression (CD326) in cancer: A short review. *Cancer Treat Rev*. 2012;38(1):68-75.

92. Litvinov SV, Balzar M, Winter MJ, Bakker HAM, Bruijn IHB-d, Prins F, et al. Epithelial Cell Adhesion Molecule (Ep-CAM) Modulates Cell–Cell Interactions Mediated by Classic Cadherins. *J Cell Biol*. 1997;139(5):1337-48.

93. Balzar M, Winter MJ, de Boer CJ, Litvinov SV. The biology of the 17–1A antigen (Ep-CAM). *J Mol Med*. 1999;77(10):699-712.

94. Munz M, Baeuerle PA, Gires O. The Emerging Role of EpCAM in Cancer and Stem Cell Signaling. *Cancer Res*. 2009;69(14):5627-9.

95. Trzpis M, McLaughlin PMJ, de Leij LMFH, Harmsen MC. Epithelial Cell Adhesion Molecule: More than a Carcinoma Marker and Adhesion Molecule. *Am J Pathol*. 2007;171(2):386-95.

96. Maetzel D, Denzel S, Mack B, Canis M, Went P, Benk M, et al. Nuclear signalling by tumour-associated antigen EpCAM. *Nat Cell Biol*. 2009;11(2):162-71.

97. Münz M, Kieu C, Mack B, Schmitt B, Zeidler R, Gires O. The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. *Oncogene*. 2004;23(34):5748–58.

98. Maaser K, Borlak J. A genome-wide expression analysis identifies a network of EpCAM-induced cell cycle regulators. *Br J Cancer*. 2008;99(10).

99. Gires O, Stoecklein NH. Dynamic EpCAM expression on circulating and disseminating tumor cells: causes and consequences. *Cell Mol Life Sci*. 2014;71(22):4393-402.

100. Rao CG, Chianese D, Doyle GV, Miller MC, Russell T, Sanders RA, et al. Expression of epithelial cell adhesion molecule in carcinoma cells present in blood and primary and metastatic tumors. *Int J Oncol*. 2005;27(1):49-57.

101. Punnoose EA, Atwal SK, Spoerke JM, Savage H, Pandita A, Yeh R-F, et al. Molecular Biomarker Analyses Using Circulating Tumor Cells. *PLoS ONE*. 2010;5(9):e12517.

102. Gorges T, Tinhofer I, Drosch M, Rose L, Zollner T, Krahn T, et al. Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. *BMC Cancer*. 2012;12(1):178.

103. Gires O, Kieu C, Fix P, Schmitt B, Münz M, Wollenberg B, et al. Tumor necrosis factor α negatively regulates the expression of the carcinoma-associated antigen epithelial cell adhesion molecule. *Cancer*. 2001;92(3):620-8.

104. Flieger D, Hoff AS, Sauerbruch T, Schmidt-Wolf IGH. Influence of cytokines, monoclonal antibodies and chemotherapeutic drugs on epithelial cell adhesion molecule (EpCAM) and Lewis(Y) antigen expression. *Clin Exp Immunol*. 2001;123(1):9-14.

105. Spizzo G, Gastl G, Obrist P, Fong D, Haun M, Grünewald K, et al. Methylation status of the Ep-CAM promoter region in human breast cancer cell lines and breast cancer tissue. *Cancer Lett*. 2006;246(1-2):253-61.

106. Alix-Panabieres C, Pantel K. Challenges in circulating tumour cell research. *Nat Rev Cancer*. 2014;14(9):623-31.

107. Campos M, Luque R, Jiménez J, Martínez R, Warleta F, Sánchez-Quesada C, et al. Simultaneous phenotypic and genetic characterization of single circulating tumor cells from colon cancer patients. *Histol Histopathol*. 2013;28:1439-50.

108. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-Throughput Droplet Digital PCR System for Absolute Quantitation of DNA Copy Number. *Anal Chem*. 2011;83(22):8604-10.

109. Guttery DS, Page K, Hills A, Woodley L, Marchese SD, Rghebi B, et al. Noninvasive Detection of Activating Estrogen Receptor 1 (ESR1) Mutations in Estrogen Receptor–Positive Metastatic Breast Cancer. *Clin Chem*. 2015;61(7):974-82.

110. Neves RP, Raba K, Schmidt O, Honisch E, Meier-Stiegen F, Behrens B, et al. Genomic High-Resolution Profiling of Single CKpos/CD45neg Flow-Sorting Purified Circulating Tumor Cells from Patients with Metastatic Breast Cancer. *Clin Chem*. 2014;60(10):1290-7.

111. Heitzer E, Auer M, Gasch C, Pichler M, Ulz P, Hoffmann EM, et al. Complex Tumor Genomes Inferred from Single Circulating Tumor Cells by Array-CGH and Next-Generation Sequencing. *Cancer Res*. 2013;73(10):2965-75.

112. Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nat Meth*. 2006;3(7):551-9.

113. Chimonidou M, Strati A, Tzitzira A, Sotiropoulou G, Malamos N, Georgoulias V, et al. DNA Methylation of Tumor Suppressor and Metastasis Suppressor Genes in Circulating Tumor Cells. *Clin Chem*. 2011;57:1169-77.

114. Stathopoulou A, Vlachonikolis I, Mavroudis D, Perraki M, Kouroussis C, Apostolaki S, et al. Molecular Detection of Cytokeratin-19-Positive Cells in the Peripheral Blood of Patients With Operable Breast Cancer: Evaluation of Their Prognostic Significance. *J Clin Oncol*. 2002;20(16):3404-12.

115. Xenidis N, Perraki M, Kafousi M, Apostolaki S, Bolonaki I, Stathopoulou A, et al. Predictive and Prognostic Value of Peripheral Blood Cytokeratin-19 mRNA-Positive Cells Detected by Real-Time Polymerase Chain Reaction in Node-Negative Breast Cancer Patients. *J Clin Oncol*. 2006;24(23):3756-62.

116. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat Rev Cancer*. 2008;8(5):329-40.

117. Andreopoulou E, Yang LY, Rangel KM, Reuben JM, Hsu L, Krishnamurthy S, et al. Comparison of assay methods for detection of circulating tumor cells in metastatic breast cancer: AdnaGen AdnaTest BreastCancer Select/Detect™ versus Veridex CellSearch™ system. *Int J Cancer*. 2012;130(7):1590-7.

118. LeBleu VS, O'Connell JT, Gonzalez Herrera KN, Wikman H, Pantel K, Haigis MC, et al. PGC-1α mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. *Nat Cell Biol*. 2014;16(10):992-1003.

119. Powell AA, Talasaz AH, Zhang H, Coram MA, Reddy A, Deng G, et al. Single cell profiling of Circulating tumor cells: Transcriptional heterogeneity and diversity from breast cancer cell lines. *PLoS ONE*. 2012;7(5):e33788.

120. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, et al. Circulating Tumor Cell Clusters Are Oligoclonal Precursors of Breast Cancer Metastasis. *Cell*. 2014;158(5):1110-22.

121. Ting DT, Wittner BS, Ligorio M, Vincent Jordan N, Shah AM, Miyamoto DT, et al. Single-Cell RNA Sequencing Identifies Extracellular Matrix Gene Expression by Pancreatic Circulating Tumor Cells. *Cell Reports*. 2014;8(6):1905-18.

122. Payne RE, Wang F, Su N, Krell J, Zebrowski A, Yagüe E, et al. Viable circulating tumour cell detection using multiplex RNA in situ hybridisation predicts progression-free survival in metastatic breast cancer patients. *Br J Cancer*. 2012;106(11):1790-7.

123. Wang F, Flanagan J, Su N, Wang L-C, Bui S, Nielson A, et al. RNAscope: A Novel in Situ RNA Analysis Platform for Formalin-Fixed, Paraffin-Embedded Tissues. *J Mol Diagn*. 2012;14(1):22-9.

124. Gasch C, Plummer PN, Jovanovic L, McInnes LM, Wescott D, Saunders CM, et al. Heterogeneity of miR-10b expression in circulating tumor cells. *Sci Rep*. 2015;5:15980.

125. Fehm T, Braun S, Muller V, Janni W, Gebauer G, Marth C, et al. A concept for the standardized detection of disseminated tumor cells in bone marrow from patients with primary breast cancer and its clinical implementation. *Cancer*. 2006;107(5):885-92.

126. Fehm T, Solomayer EF, Meng S, Tucker T, Lane N, Wang J, et al. Methods for isolating circulating epithelial cells and criteria for their classification as carcinoma cells. *Cytotherapy*. 2005;7(2):171-85.

127. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating Tumor Cells, Disease Progression, and Survival in Metastatic Breast Cancer. *New Engl J Med*. 2004;351(8):781-91.

128. Mego M, Mani SA, Lee B-N, Li C, Evans KW, Cohen EN, et al. Expression of Epithelial-Mesenchymal Transition-Inducing Transcription Factors in Primary Breast Cancer: The Effect of Neoadjuvant Therapy. *Int J Cancer*. 2012;130(4):808-16.

129. Barrière G, Riouallon A, Renaudie J, Tartary M, Rigaud M. Mesenchymal and stemness circulating tumor cells in early breast cancer diagnosis. *BMC Cancer*. 2012;12:114.

130. Raimondi C, Gradilone A, Naso G, Vincenzi B, Petracca A, Nicolazzo C, et al. Epithelial-mesenchymal transition and stemness features in circulating tumor cells from breast cancer patients. *Breast Cancer Res Treat*. 2011;130(2):449-55.

131. Armstrong AJ, Marengo MS, Oltean S, Kemeny G, Bitting RL, Turnbull J, et al. Circulating Tumor Cells from Patients with Advanced Prostate and Breast Cancer Display Both Epithelial and Mesenchymal Markers. *Mol Cancer Res*. 2011;9(8):997-1007.

132. Giordano A, Giuliano M, De Laurentiis M, Arpino G, Jackson S, Handy BC, et al. Circulating tumor cells in immunohistochemical subtypes of metastatic breast cancer: lack of prediction in HER2-positive disease treated with targeted therapy. *Ann Oncol*. 2012;23(5):1144-50.

133. Mego M, Gao H, Lee B-N, Cohen EN, Tin S, Giordano A, et al. Prognostic Value of EMT-Circulating Tumor Cells in Metastatic Breast Cancer Patients Undergoing High-Dose Chemotherapy with Autologous Hematopoietic Stem Cell Transplantation. *J Cancer*. 2012;3:369-80.

134. Aktas B, Tewes M, Fehm T, Hauch S, Kimmig R, Kasimir-Bauer S. Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients. *Breast Cancer Res*. 2009;11(4):R46.

135. Giordano A, Gao H, Anfossi S, Cohen E, Mego M, Lee B-N, et al. Epithelial-Mesenchymal Transition and Stem Cell Markers in Patients with HER2-Positive Metastatic Breast Cancer. *Mol Cancer Ther*. 2012;11(11):2526-34.

136. Zhang L, Ridgway LD, Wetzel MD, Ngo J, Yin W, Kumar D, et al. The Identification and Characterization of Breast Cancer CTCs Competent for Brain Metastasis. *Sci Transl Med*. 2013;5(180):180ra48.

137. Yu M, Bardia A, Aceto N, Bersani F, Madden MW, Donaldson MC, et al. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. *Science*. 2014;345(6193):216-20.

138. Cayrefourcq L, Mazard T, Joosse S, Solassol J, Ramos J, Assenat E, et al. Establishment and Characterization of a Cell Line from Human Circulating Colon Cancer Cells. *Cancer Res*. 2015;75(5):892-901.

139. Baccelli I, Schneeweiss A, Riethdorf S, Stenzinger A, Schillert A, Vogel V, et al. Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. *Nat Biotechnol*. 2013;31(6):539-44.

140. Hodgkinson CL, Morrow CJ, Li Y, Metcalf RL, Rothwell DG, Trapani F, et al. Tumorigenicity and genetic profiling of circulating tumor cells in small-cell lung cancer. *Nat Med*. 2014;20(8):897-903.

141. Rack B, Schindlbeck C, Jückstock J, Andergassen U, Hepp P, Zwingers T, et al. Circulating Tumor Cells Predict Survival in Early Average-to-High Risk Breast Cancer Patients. *J Natl Cancer Inst*. 2014;106(5):dju066.

142. Molloy TJ, Bosma AJ, Baumbusch LO, Synnestvedt M, Borgen E, Russnes HG, et al. The prognostic significance of tumour cell detection in the peripheral blood versus the bone marrow in 733 early-stage breast cancer patients. *Breast Cancer Res*. 2011;13(3):R61.

143. Zhang L, Riethdorf S, Wu G, Wang T, Yang K, Peng G, et al. Meta-Analysis of the Prognostic Value of Circulating Tumor Cells in Breast Cancer. *Clin Cancer Res*. 2012;18(20):5701-10.

144. Pierga JY, Hajage D, Bachelot T, Delaloge S, Brain E, Campone M, et al. High independent prognostic and predictive value of circulating tumor cells compared with

serum tumor markers in a large prospective trial in first-line chemotherapy for metastatic breast cancer patients. *Ann Oncol*. 2012;23(3):618-24.

145. Wallwiener M, Hartkopf A, Baccelli I, Riethdorf S, Schott S, Pantel K, et al. The prognostic impact of circulating tumor cells in subtypes of metastatic breast cancer. *Breast Cancer Res Treat*. 2013;137(2):503-10.

146. Bidard F-C, Peeters DJ, Fehm T, Nolé F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. *Lancet Oncol*. 2014;15(4):406-14.

147. Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, et al. Circulating Tumor Cells and Response to Chemotherapy in Metastatic Breast Cancer: SWOG S0500. *J Clin Oncol*. 2014;32(31):3483-9.

148. Bidard FC, Fehm T, Ignatiadis M, Smerage JB, Alix-Panabieres C, Janni W, et al. Clinical application of circulating tumor cells in breast cancer: overview of the current interventional trials. *Cancer Metastasis Rev*. 2013;13:179-88.

149. Wülfing P, Borchard J, Buerger H, Heidl S, Zänker KS, Kiesel L, et al. HER2-Positive Circulating Tumor Cells Indicate Poor Clinical Outcome in Stage I to III Breast Cancer Patients. *Clin Cancer Res*. 2006;12(6):1715-20.

150. Ignatiadis M, Xenidis N, Perraki M, Apostolaki S, Politaki E, Kafousi M, et al. Different Prognostic Value of Cytokeratin-19 mRNA positive Circulating Tumor Cells According to Estrogen Receptor and HER2 Status in Early-Stage Breast Cancer. *J Clin Oncol*. 2007;25(33):5194-202.

151. Slade MJ, Payne R, Riethdorf S, Ward B, Zaidi SAA, Stebbing J, et al. Comparison of bone marrow, disseminated tumour cells and blood-circulating tumour cells in breast cancer patients after primary treatment. *Br J Cancer*. 2009;100(1):160-6.

152. Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet*. 2005;365(9472):1687-717.

153. Krawczyk N, Banys M, Hartkopf A, Hagenbeck C, Melcher C, Fehm T. Circulating tumour cells in breast cancer. *Ecancermedicalscience*. 2013;7:352.

154. Saloustros E, Perraki M, Apostolaki S, Kallergi G, Xyrafas A, Kalbakis K, et al. Cytokeratin-19 mRNA-positive circulating tumor cells during follow-up of patients with operable breast cancer: prognostic relevance for late relapse. *Breast Cancer Res*. 2011;13(3):R60.

155. Boutrus RR, Abi Raad RF, Kuter I, Ancukiewicz M, Roberts L, Solomon N, et al. Circulating tumor cells as predictors of response and failure in breast cancer patients treated with preoperative chemotherapy. *Int J Biol Markers*. 2013;28(1):17-23.

156. Liu MC, Shields PG, Warren RD, Cohen P, Wilkinson M, Ottaviano YL, et al. Circulating Tumor Cells: A Useful Predictor of Treatment Efficacy in Metastatic Breast Cancer. *J Clin Oncol*. 2009;27(31):5153-9.

157. Budd GT, Cristofanilli M, Ellis MJ, Stopeck A, Borden E, Miller MC, et al. Circulating Tumor Cells versus Imaging—Predicting Overall Survival in Metastatic Breast Cancer. *Clin Cancer Res*. 2006;12(21):6403-9.

158. Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, et al. Circulating Tumor Cells at Each Follow-up Time Point during Therapy of Metastatic Breast Cancer Patients Predict Progression-Free and Overall Survival. *Clin Cancer Res*. 2006;12(14):4218-24.

159. Nakamura S, Yagata H, Ohno S, Yamaguchi H, Iwata H, Tsunoda N, et al. Multi-center study evaluating circulating tumor cells as a surrogate for response to treatment and overall survival in metastatic breast cancer. *Breast Cancer*. 2010;17(3):199-204.

160. Hartkopf AD, Wagner P, Wallwiener D, Fehm T, Rothmund R. Changing Levels of Circulating Tumor Cells in Monitoring Chemotherapy Response in Patients with Metastatic Breast Cancer. *Anticancer Res*. 2011;31(3):979-84.

161. Azim HA, Agbor-tarh D, Bradbury I, Dinh P, Baselga J, Di Cosimo S, et al. Pattern of Rash, Diarrhea, and Hepatic Toxicities Secondary to Lapatinib and Their Association With Age and Response to Neoadjuvant Therapy: Analysis From the NeoALTTO Trial. *J Clin Oncol*. 2013;31(36):4504-11.

162. Xenidis N, Perraki M, Apostolaki S, Agelaki S, Kalbakis K, Vardakis N, et al. Differential effect of adjuvant taxane-based and taxane-free chemotherapy regimens on the CK-19 mRNA-positive circulating tumour cells in patients with early breast cancer. *Br J Cancer*. 2013;108(3):549-56.

163. Fehm T, Krawczyk N, Solomayer E-F, Becker-Pergola G, Durr-Storzer S, Neubauer H, et al. ERalpha-status of disseminated tumour cells in bone marrow of primary breast cancer patients. *Breast Cancer Res*. 2008;10(5):R76.

164. Krawczyk N, Banys M, Neubauer H, Solomayer EF, Gall C, Hahn M, et al. HER2 Status on Persistent Disseminated Tumor Cells after Adjuvant Therapy May Differ from Initial HER2 Status on Primary Tumor. *Anticancer Res*. 2009;29(10):4019-24.

165. Aktas B, Müller V, Tewes M, Zeitz J, Kasimir-Bauer S, Loehberg CR, et al. Comparison of estrogen and progesteronereceptor status of circulating tumor cells and the primary tumor in metastatic breast cancer patients. *Gynecol Oncol*. 2011;122(2):356-60.

166. McInnes LM, Jacobson N, Redfern A, Dowling A, Thompson EW, Saunders CM. Clinical Implications of Circulating Tumor Cells of Breast Cancer Patients: Role of Epithelial–Mesenchymal Plasticity. *Front Oncol*. 2015;5:42.

167. Rack B, Jückstock J, Günthner-Biller M, Andergassen U, Neugebauer J, Hepp P, et al. Trastuzumab clears HER2/neu-positive isolated tumor cells from bone marrow in primary breast cancer patients. *Arch Gynecol Obstet*. 2012;285(2):485-92.

168. Hong B, Zu Y. Detecting Circulating Tumor Cells: Current Challenges and New Trends. *Theranostics*. 2013;3(6):377-94.

2 Aim of the study

Until now, the detection and evaluation of relevant therapeutic targets in breast cancer treatment is based on primary tumor characteristics. However, primary tumors are extremely heterogeneous. They harbor cellular subsets that may differ in their metastatic potential and in sensitivity to different chemotherapeutic substances, potentially leading to treatment failure. CTCs are considered as surrogates of minimal residual disease and their molecular characterization provides a key to unravel their cellular origin as well as novel treatment paradigms, consequently allowing the stratification of patients and real-time monitoring of systemic therapies. So far, much of what is known about CTCs in metastatic breast cancer simply relates to their quantification and its association with prognosis.

The aim of this study was to make a decisive contribution to the optimization of liquid biopsies by improving the enrichment of EpCAM-negative CTC subsets and subsequently, the molecular characterization/mutational analysis of CTCs: both of which are important for the development of personalized treatments to circumvent therapy resistance. To this end, the BMBF-supported project “CTC detect: Nicht-invasiver Diagnostikansatz zur Bestimmung der Patientengefährdung durch Tumor-Metastasierung basierend auf zirkulierenden Tumorzellen in Blut und abgeleiteter Marker“ (MicroTec Südwest, grant number 16SV5122) was launched, in which the primary aim was to develop an automated device that can quantify CTCs via label-free optical detection and measure clinically relevant tumor markers in parallel. Additionally, methods that allow the detection, isolation and characterization of single EpCAM-low or -negative tumor cells should be established. Furthermore, it was planned to develop a chip in a microarray format as a multi-marker test combining different capture molecules facilitating the identification, analysis and phenotyping of breast cancer cell lines and breast cancer clinical samples. The second part of the study aimed to analyze HER2-positive CTCs from patients with primarily HER2-negative metastatic breast cancer for activating *PIK3CA* mutations which might play a role in the resistance to HER2-targeted treatment. This should be achieved within a translational project associated to the DETECT III study. A workflow regarding CTC enrichment, DNA isolation, whole genome amplification and *PIK3CA*-specific PCR should be established, finally enabling the mutational analysis for *PIK3CA* hotspot mutations E542K/E545K and H1047R. Therein, mutations were ought to be detected via the already published SNaPshot assay. Furthermore, the workflow should be adapted to allow single cell mutational analysis. In the future, the information on the presence of activating *PIK3CA* mutations in CTCs might be correlated to treatment response and can contribute to the early adjustment of individual therapies.

3 Manuscripts

3.1 Original Research Articles

3.1.1 Analysing the mutational status of PIK3CA in circulating tumor cells from metastatic breast cancer patients

Original Research Article in ***Molecular Oncology***

Authorship:	first author (shared)
Impact factor:	5.935
Status:	published; *Mol Onc* 2013 Oct;7(5):976-86
DOI:	10.1016/j.molonc.2013.07.007
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Contribution in %:	45%
Own contribution:	Designed and conceived the experiments, performed the experiments, analyzed and interpreted the data, conceived and wrote the publication

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Analysing the mutational status of *PIK3CA* in circulating tumor cells from metastatic breast cancer patients

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ABSTRACT

The frequently altered phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway is involved in the regulation of cellular processes required for breast carcinogenesis. The aim of the project was to develop a method to identify hotspot mutations in the *PIK3CA* gene in circulating tumor cells (CTCs) of metastatic breast cancer (metBC) patients.

From 44 enrolled CTC-positive metBC patients a total number of 57 peripheral blood samples were analysed by CellSearch®. Genomic DNA of enriched CTCs was isolated, amplified and analyzed for *PIK3CA* mutations in exons 9 and 20 which lead to E542K, E545K or H1047R amino acid changes and result in increased PI3K activity. The mutations were detected by using SNaPshot-methodology comprising PCR amplification and single nucleotide primer extension.

SNaPshot analysis was established using genomic DNA from different breast cancer cell lines and then successfully transferred to investigate blood samples and single cells. Overall, twelve hotspot mutations in either exon 9/E545K (6/12, 50%) or exon 20/H1047R (6/12, 50%) could be determined within 9 out of 57 (15.8%) blood samples from 7 out of 44 (15.9%) patients; CTC counts ranged from 1 to 9748. *PIK3CA* variants E542K, E545G and E545A were not detected.

Analysing the *PIK3CA* genotype of CTCs has clinical relevance with respect to drug resistance, e.g. against HER2-targeted therapy. The herein described approach including SNaPshot technology provides a simple method to characterize hotspot mutations within CTCs

Abbreviations: APC, allophycocyanin; CK-PE, cytokeratin-phycoerythrin; CS, CellSearch®; CTC(s), circulating tumor cell(s); DAPI, 4,6-diamidino-2-phenylindole; DTCs, disseminated tumor cells; ER, estrogen receptor; FITC, fluorescein-isothiocyanate; HER2, human epidermal growth factor receptor 2; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog; PR, progesterone receptor; SNP, single nucleotide polymorphism; WGA, Whole Genome Amplification.

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enriched from peripheral blood and can be easily adopted for analysing further therapeutically relevant SNPs.

1. Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008 (Jemal et al., 2010, 2011).

Up to now, breast cancer treatment decisions are based upon the histology of the primary tumor and its expression status of molecular markers such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Although significant improvements in breast cancer treatment have been achieved within the last decades, the need for novel therapeutic approaches aiming at specific tumorigenic cells and their key oncogenic pathways has become obvious.

In several clinical studies, the detection and enumeration of CTCs from breast cancer patients has been established showing a correlation with decreased progression-free and overall survival in operable (Ignatiadis et al., 2008; Xenidis et al., 2006) and advanced breast cancer (Cristofanilli et al., 2005). Molecular characterization of CTCs is essential to confirm their malignant origin and to identify diagnostically and therapeutically relevant targets which help to stratify cancer patients for individual therapies (Scher et al., 2009).

Breast cancer cells in primary tumors, CTCs as well as disseminated tumor cells (DTCs) vary phenotypically and functionally in established prognostic factors such as HER2, ER, PR and in gene expression profiles (Bozionellou et al., 2004; Meng et al., 2004; Müller and Pantel, 2009; Strati et al., 2011; Powell et al., 2012). These findings advocate for the usage of CTCs as 'liquid biopsy' to refine therapeutic regimens.

In breast cancer, the PI3K/PTEN/AKT pathway which is involved in the regulation of central cellular processes is often dysregulated, due to alterations in several pathway components including amplification of the *HER2* gene locus, loss of PTEN (phosphatase and tensin homolog) protein function, and mutations of the *PIK3CA* gene (Bachman et al., 2004; Dunlap et al., 2010; Paradiso et al., 2007). *PIK3CA* gene variations result in different activation levels of PI3K (Araújo et al., 2010).

Class 1A PI3-kinases are heterodimeric lipid kinases composed of a catalytic subunit (p110a), encoded by the *PIK3CA* gene and a regulatory subunit (p85a). Specific mutations within *PIK3CA* that cluster in hotspots located in exon 9 (E542/545K; catalytic domain) and exon 20 (H1047R; kinase domain) have been demonstrated to activate PI3K/AKT signaling and provide a strong selective growth advantage to the cell. This suggestion is accompanied by the fact that all three hotspot mutations confer *in vitro* and *in vivo* tumorigenicity (Bader et al., 2006; Ikenoue et al., 2005; Isakoff et al., 2005; Kang et al., 2005; Samuels et al., 2005; Zhao et al., 2005). These observations and the fact that specific inhibitors for PIK3CA are being developed emphasize the importance of analyzing the *PIK3CA* mutational status in CTCs, and relating this status to potential drug resistance, especially in those CTCs surviving HER2-targeting therapy.

In order to detect *PIK3CA* mutations, PCR-based screening methods and direct sequencing of PCR products have been applied (Board et al., 2008; Simi et al., 2008; Qiu et al., 2008). Here for the first time, we describe the application of a modified approach based on the SNaPshot-technology published by Hurst et al. in order to simultaneously analyse the *PIK3CA* mutations E542K, E545 G/K and H1047R in CTCs of metBC patients and in single breast cancer cells. This assay allows a combined implementation of multiplex PCR amplification and multiplex primer extension for the targeted detection of several mutations in one approach (Hurst et al., 2009). We have used this technology because it enables a sensitive analysis of SNPs from single tumor cells in a multiplex approach.

2. Material and methods

2.1. Cell lines and cell culture

MCF-7, SKBR-3 and T47D breast cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). SKBR-3 cells were reported to contain a *PIK3CA* wild-type gene, while MCF-7 and T47D cells harbor heterozygous hotspot mutations in the *PIK3CA* gene: MCF-7 is mutated in exon 9 (E545K), T47D in exon 20 (H1047R) (Hollestelle et al., 2007; Jensen et al., 2011; Kataoka et al., 2010; Weigelt et al., 2011). All cell lines were cultured in RPMI 1640 containing 10% fetal calf serum and 1% Penicillin-Streptomycin (all Gibco, Karlsruhe, Germany). Culture medium for MCF-7 was supplemented with 25 mM HEPES (Gibco); for T47D cells 10 mM HEPES, 1 mM sodium pyruvate (Gibco) and 0.45% D-(+)Glucose solution (Sigma–Aldrich, Munich, Germany) was added. All cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. For spiking experiments, dilution series for MCF-7, SKBR-3 and T47D cells were performed and about 100 cells in PBS were spiked into 7.5 ml blood of healthy volunteers collected into CellSearch® (CS) CellSave preservative tubes (Veridex LLC, Raritan, NJ, USA).

2.2. Patients

Patients were recruited within a translational spin-off project of the German Detect III trial which is investigating blood samples from primarily HER2-negative metastatic breast cancer patients who harbor HER2-positive CTCs (for more information see www.detect-studien.de). In total, 57 blood samples (7.5 ml) from 44 CTC-positive advanced metBC patients were collected into CellSave tubes and processed by CellSearch® (Veridex LLC, Raritan, NJ, USA). The clinical patient data are shown in Table 1, more detailed information is provided in the Supplemental data Table 1. Written informed consent

Table 1 – Clinical data of patients (primary tumor (PT) and CTCs).

Characteristics		Total	In %
Patients		44	100.0
Age			
Mean	58.4		
Median	59.0		
Range	26–78		
Tumor size			
T1		13	29.5
T2		15	34.1
T3		7	15.9
T4		8	18.2
Tx		1	2.3
Nodal status			
Negative		11	25.0
Positive		29	65.9
Nx		4	9.1
M status of primary tumor at the time of diagnosis			
M0		29	65.9
M1		7	15.9
Mx		8	18.2
Histology			
Ductal		33	75.0
Lobular		7	15.9
Others		4	9.1
Grading			
GI		0	0
GII		18	40.9
GIII		21	47.7
Not determined		5	11.4
ER status			
Positive		30	68.2
Negative		12	27.3
Not determined		2	4.5
PR status			
Positive		28	63.6
Negative		14	31.8
Not determined		2	4.5
HER2 status			
Negative		36	81.8
Not determined		8	18.2
Patients with HER2+ CTC		15	34.1
Range of CTC	1–9748		

was obtained from all participating patients and the study was approved by the local ethical committee (525/2011AMG1).

2.3. *Blood sample processing, detection of CTCs and assessment of HER2 expression*

Isolation and enumeration of tumor cells from spiked blood samples obtained from healthy volunteers as well as from blood of metBC patients were processed by using the FDA-approved CS. This system allows for the automated enrichment and immunostaining of CTCs in patients with metastatic breast, colon and prostate cancer. A total of 7.5 ml of whole blood was collected in a CellSave Preservative Tube and the CellSearch® Circulating Tumor Cell Kit (Veridex LLC, Raritan, NJ, USA) was applied for CTC enrichment and enumeration. Herein, immunomagnetic enrichment is achieved by using an anti-EpCAM ferrofluid. Enriched cells are then fluorescently stained with the nucleic acid dye 4,6-diamidino-2-phenylindole (DAPI) and labeled with monoclonal antibodies specific for epithelial cells (anti-cytokeratins 8, 18, 19) and leukocytes (anti-CD45). CTCs are subsequently identified by cytokeratin positivity/negativity for the leukocyte common antigen CD45 and nuclear DAPI staining to ensure the integrity of the nucleus. All CTC assessments were performed by trained and Veridex certified technicians. Blood samples were designated as positive when at least one CTC was present. HER2 expression of CTCs was characterized within the CS by the addition of a fluorescein-labeled anti-HER2 antibody (CS tumor phenotyping reagent HER2; Veridex), as described previously (Riethdorf et al., 2010). HER2-specific immunostaining of CTCs was categorized into negative (0), weak (1+), moderate (2+) and strong (3+) (Riethdorf et al., 2010), whereas only HER2-strong expressing CTCs were counted as HER2-positive. Further blood sample processing after CTC/HER2 assessment can be found depicted in Figure 1.

2.4. *Extraction of genomic DNA and Whole Genome Amplification (WGA)*

DNA extraction from CS enriched tumor cells was obtained by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Therefore, the entire contents of CTC-positive CS cartridges were transferred into tubes, digested with Proteinase K and complete cell lysis was verified by visual control under a fluorescence microscope. Column-bound DNA was eluted with 30 µl of nuclease-free water and concentrated further to a final volume of 10 µl. 3 µl were employed for WGA using the Ampli1™ WGA Kit from Silicon Biosystems according to manufacturer's protocol. The WGA procedure is based on a ligation-mediated PCR following a site-specific DNA digestion. For quality control, 2 µl of Ampli1™ products were analysed utilising the Ampli1™ QC Kit (Silicon Biosystems, Bologna, Italy).

2.5. PCR

After WGA, the first gene-specific PCR for *PIK3CA* exons 9 and 20 was performed in a volume of 25 µl containing 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 5% DMSO, 1U Taq DNA polymerase (Life Technologies GmbH, Darmstadt, Germany) and 5 µl of whole genome amplified DNA as template. Primers were designed by Beckman Coulter to produce amplicons covering hotspot codons E542, E545 (exon 9) and H1047 (exon 20) (0.2 µM each; Table 2). Thermal cycler conditions were: 95 °C for 5min, 45 cycles of 95 °C for 45sec, 58 °C for 45sec, 72 °C for 45sec and finally 10min at 72 °C. PCR products were checked for quality and quantity by running 5 µl in a 3% agarose-TBA gel. Remaining PCR products were purified using a PCR Purification Kit (Qiagen). Purified product (2.5 µl) was then further processed for a second exon 9 and exon 20 specific PCR ("nested PCR") using nested PCR primers (Table 2) with the same PCR conditions as described above, only the number of PCR cycles was increased to 60.

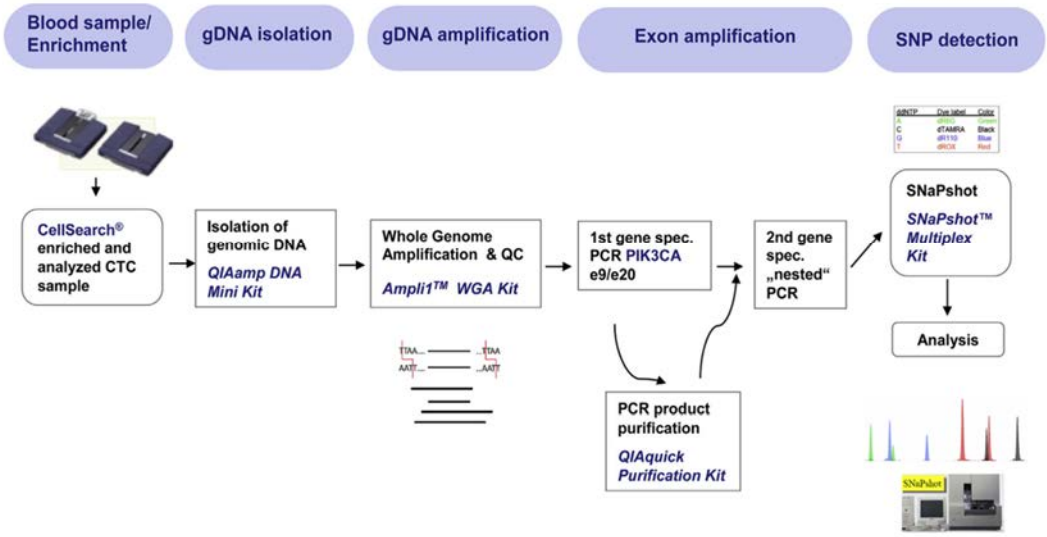

Figure 1 – Workflow for the molecular characterization of circulating tumor cells by dint of CTC enrichment, Whole Genome Amplification and SNaPshot technology for *PIK3CA*.

2.6. *SNaPshot assay*

The probes used in the SNaPshot reaction were adopted from Hurst et al. (2009) (Table 2). Probes anneal one nucleotide position 5′ of the potential mutated nucleotide on the template DNA and are extended by one base only due to the use of dideoxynucleotides (ddNTPs). Each of the four ddNTPs (ddATP, ddCTP, ddGTP, ddTTP) is labeled with a different fluorophore enabling them to be distinguished from each other. SNaPshot analysis was performed using the SNaPshot Multiplex Kit (Applied Biosystems, Carlsbad, US). Reactions were conducted in a total volume of 10 μl containing 4 μl PCR product (2 μl for each exon), 5 μl SNaPshot Multiplex Master Mix, and 1 μl of pooled SNaPshot probes (0.8 μM E542K, 2.3 μM E545G, 1.5 μM E545K, 1.5 μM H1047R). Primer extension was carried out in a thermal cycler (MJ Research, Watertown, US) using the following program: 25 cycles of 96 °C, 10sec (denaturation); 50 °C, 5sec (probe-annealing) and 60 °C, 30sec (primer extension), then cooling down to 4 °C. In order to eliminate unincorporated ddNTPs, labeled extension products were treated with 0.5 μl Calf Intestinal Phosphatase (1 U/μl, NEB, Ipswich, MA) and incubated for 1 h at 37 °C, followed by a denaturation step at 75 °C for 15min. Subsequently, 10 μl of HiDi™ formamide and 0.6 μl of an internal size standard (Genescan-120LIZ, both Applied Biosystems) were mixed with 2 μl extension product and transferred to an ABgene Thermo-Fast 96 PCR Detection Plate (Thermo Scientific, Karlsruhe, Germany) for separation using

Table 2 – Primer sequences for first gene-specific, nested PCR amplification and for SNaPshot analysis[a] for exon 9 and exon 20 of *PIK3CA*.

PIK3CA exon	Primer name	Sequence (5'–>3')	Product size (bp)
9	e9_131_left	GCTAGAGACAATGAATTAAGGGAAAA	131
	e9_131_right	CTCCATTTTAGCACTTACCTGTGAC	
20	e20_167_left	GCCAGAACTACAATCTTTTGATGAC	167
	e20_167_right	CATGCTGTTTAATTGTGTGGAAG	
9	e9_nested_left	GACAAAGAACAGCTCAAAGCAA	110
	e9_nested_right	ATTTTAGCACTTACCTGTGAC	
20	e20_nested_left	TTGATGACATTGCATACATTCG	134
	e20_nested_right	GTGGAAGATCCAATCCATTT	
PIK3CA exon	**Mutation**	**Sequence (5'–>3')[a]**	**Size (bp)**
9	E542K	$T_{(19)}$TACACGAGATCCTCTCTCT	38
	E545G	$T_{(29)}$TCCTCTCTCTGAAATCACTG	49
	E545K	$T_{(34)}$ATCCTCTCTCTGAAATCACT	54
20	H1047R	$T_{(46)}$TGAAACAAATGAATGATGCAC	67

a Primer sequences were published before (Hurst et al., 2009).

an ABI PRISM 3100 Genetic Analyzer with a 36 cm length capillary and POP-7™ polymer. SNP-analysis was performed using GeneMapper 3.7 Software.

2.7. Deposition and preparation of single breast cancer cells

In order to amplify DNA in a low volume reaction format using single cells as DNA source AmpliGrid technology (Beckman Coulter, Munich, Germany) was applied. Single MCF-7 cells were deposited in 1× PBS onto reaction sites of an AmpliGrid slide by dint of cell sorting via a BD FACSAria™ flow cytometer. After air-drying the cells, a visual quality control was performed by inspecting Hoechst 33342 positive cells using fluorescence microscopy (Axioplan 2, Carl Zeiss GmbH, Göttingen, Germany). For DNA extraction from single cells the Beckman Coulter Cell Extraction Kit was used. The master mix for cell lysis comprises the following components: 1 µl lysis enzyme, 6 µl 10× reaction buffer and 53 µl nuclease-free H_2O. A volume of 0.75 µl was pipetted to the reaction side and the droplet was covered with 5 µl sealing solution before the loaded AmpliGrid was placed on the AmpliSpeed slide cycler (Beckman Coulter) to execute the cell extraction program (75 °C, 5min; 95 °C, 2min; RT). After cell lysis, gene-specific PCR reactions with the primers listed in Table 2 were carried out on-slide in a total volume of 1 µl. One half of the amplified products was used for quality control with the Agilent DNA 1000 kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), the other half was employed for SNaPshot analysis.

3. Results

3.1. CTC assessment and determination of HER2 expression

Since the blood samples were used to set up the technique we – in contrast to the clinical cut-off of ≥5 CTCs for metBC – considered samples CTC-positive if ≥1 CTC was detected. In this study, 44 CTC-positive metBC patients were enrolled and a total number of 57 blood samples was analysed. CTC counts ranged from 1 to 9748. CTCs were further examined for HER2 expression, with 15 out of 44 patients (34.1%) harboring at least one HER2-positive (3+) CTC. In total, 23 of the 57 analysed samples (40.3%) contained HER2-positive CTCs. For two patients (4.5%) HER2-status was not assessed. In general, CTCs within patients differed strongly in HER2 expression. Figure 2 exemplifies different HER2 expression levels (negative (0), weak (1+), moderate (2+), strong (3+)) of CTCs enriched from patient 26.

3.2. SNaPshot analysis using genomic DNA from breast cancer cell lines

Initially, the SNaPshot methodology was established using genomic DNA of breast cancer cell lines harboring different *PIK3CA* hotspot mutations of interest or carrying the wild-type gene. MCF-7 cells were chosen due to a mutation in exon 9 which results in an amino acid change at position E545 (E545K). T47D cells carry a point mutation in exon 20 leading to an amino acid change at position 1047 (H1047R). Finally, SKBR-3 cells were reported to exhibit no *PIK3CA* mutations at these positions (Hollestelle et al., 2007; Jensen et al., 2011; Kataoka et al., 2010; Weigelt et al., 2011). In the beginning, primer sets for exon amplification described in Hurst et al. (2009) were used but gave non-satisfying results within our setting. Therefore, we – in cooperation with Beckman Coulter – designed new *PIK3CA* primers to optimize DNA fragment amplification for exons 9 and 20. These primers were successfully implemented for single and duplex PCR amplification.

To detect SNPs the resulting amplicons for exons 9 and 20 were then used for *PIK3CA* SNaPshot analysis. Results are depicted in Figure 3 wherein the different colored peaks – generated by the Genemapper software – indicate which of the specific fluorescently labeled dideoxynucleotides was added during the primer extension reaction. SNPs can thus

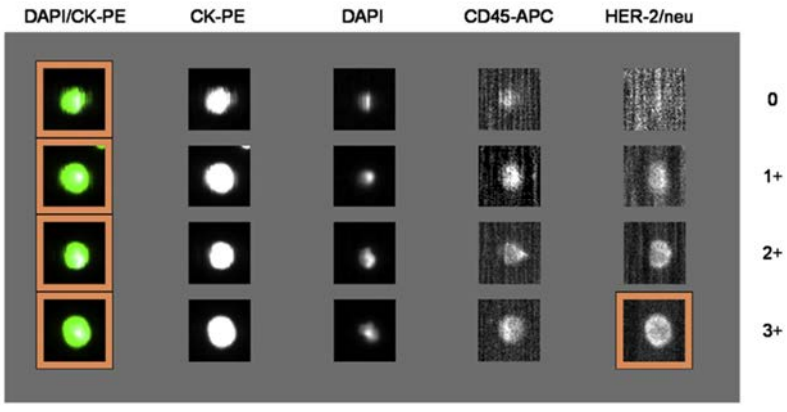

Figure 2 – HER2 immunoscoring of CTCs from patient 26. CTCs show a cytokeratin signal (CK), are DAPI-positive and CD45-negative. HER2 expression of CTCs was determined using the FITC-labeled anti-HER2 antibody in the CellSearch® system. Intensities of HER2-labeling was classified into negative (0), weak (1+), moderate (2+), and strong (3+); CK-PE, cytokeratin-phycoerythrin; DAPI, 4,6-diamidino-2-phenylindole; APC, allophycocyanin; FITC, fluorescein-isothiocyanate.

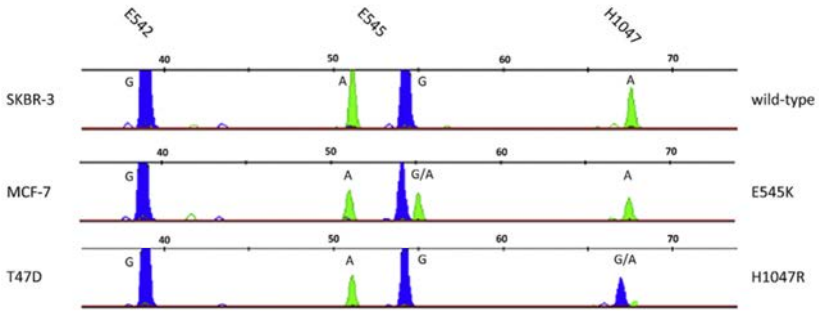

Figure 3 – SNaPshot result for detection of hotspot mutations in the *PIK3CA* gene for genomic DNA of breast cancer cell lines. SNP detection in SKBR-3 (wild-type), MCF-7 (E545K) and T47D (H1047R) performed on pooled singleplex PCRs for exon 9 and 20. Bases are represented by the following colorcode: A = green; G = blue.

be identified by the peak color and size relative to the internal Genescan-10LIZ size standard. The molecular weights of the SNaPshot extension products are influenced by the different fluorophores, thus mutant alleles are readily identified as they exhibit mobility shifts relative to the wild-type alleles. In panel 1 of Figure 3 the electropherogram obtained for SNaPshot analysis of wild-type *PIK3CA* in SKBR-3 cells is depicted. The middle and bottom panels represent the peak patterns for heterozygous mutations in exon 9/E545K (MCF-7) and exon 20/H1047R in T47D cells, respectively. To verify that SNaPshot is detecting the relevant SNPs, PCR products were sequenced (Figure 4).

3.3. SNaPshot analysis for spiked blood samples

According to the workflow depicted in Figure 1 the SNaPshot assay was then performed to detect *PIK3CA* mutations in breast cancer cell lines which were spiked into 7.5 ml of CellSave preservative blood from healthy donors. Electropherograms pictured in Figure 5 for about 100 MCF-7 and T47D cells show the aforementioned mutant alleles for E545K (MCF-7) and H1047R (T47D). Spiked SKBR-3 cells exhibit the peak pattern for wild-type *PIK3CA* at the examined positions.

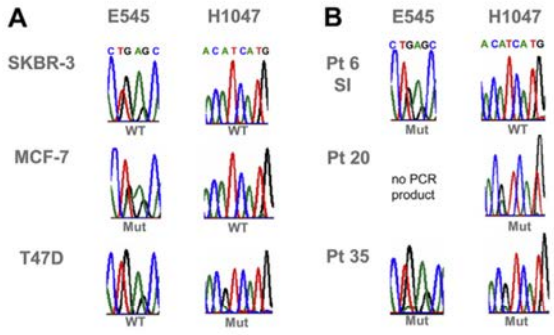

Figure 4 – Sequences for *PIK3CA* exon 9 (E545) and exon 20 (H1047) derived from nested PCR products for spiked cell lines and patient (pt) samples. A. Sequencing results for breast cancer cell lines SKBR-3 (WT), MCF-7 (E545K) and T47D (H1047R). PCR products for MCF-7 showed a heterozygous mutation at E545 where in codon GAG the G is replaced by an A resulting in AAG. Products for spiked T47D cells carried an H1047 mutation leading to CGT instead of CAT. B. Sequencing results for pts 6, 20 and 35. Sequence pattern of sample 1 (SI) from pt 6 shows a mutation in E545, the one from pt 20 harbored a mutation at position H1047, whereas amplified products of pt 35 showed both (E545K and H1047R, respectively).

3.4. SNaPshot analysis of CTCs in blood from metBC patients

Based upon the established methodology on spiked blood samples, our SNaPshot protocol was applied to CTCs in 57 blood samples obtained from 44 metBC patients (Figure 6).

For patient 6 as well as for patient 26 two independent CTC-positive blood samples were available. In both cases two different *PIK3CA* mutations, E545K and H1047R, were detected. Sample I of patient 6 (7 CTCs) exhibited an E545K mutation, and in sample II (7 CTCs) an H1047R mutation was detected. In sample I from patient 26 E545K and H1047R mutations were found simultaneously, with the H1047R mutation being detected in both - the homozygous (sample I, 345 CTCs) and heterozygous (sample II, 487 CTCs) states. For patients 18 (121 CTCs) and 27 (a, not determined; see text below, Table 3), one blood sample each was assessed revealing a mutant *PIK3CA* allele at position E545 (E545K). E545K and H1047R mutations were observed simultaneously within CTC populations from single blood samples of patients 33 and 35 (7 and 2 CTCs). Finally, for patient 20 (4950 CTCs) a heterozygous mutation in H1047R was detected, whereas the mutational analysis for exon 20 could not be conducted due to failure of nested PCR for exon 9.

Overall, twelve hotspot mutations in either exon 9/E545K (6/12, 50%) or exon 20/H1047R (6/12, 50%) of *PIK3CA* were detected in 9 out of 57 (15.8%) blood samples from 7 out of 44 (15.9%) patients (Table 3). Point mutations resulting in E542K, E545G or E545A were not detected within our study. For patient 27 the exact number of CTCs could not be determined because of ferrofluid in the CS cartridge. However, CTCs were present according to standard microscopic inspection. Mutations could be detected in blood samples with as low as two CTCs.

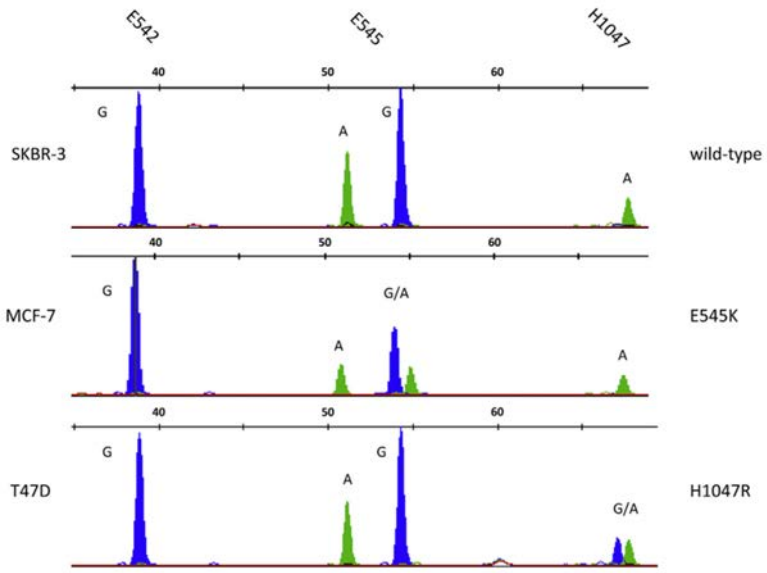

Figure 5 – SNaPshot electropherograms for detection of hotspot mutations in the *PIK3CA* gene for spiked SKBR-3, MCF-7 and T47D breast cancer cells. SNP detection in about 100 SKBR-3 (wild type), MCF-7 (E545K) and T47D (H1047R) cells performed on pooled singleplex PCRs for exon 9 and 20. Bases are represented by the following colorcode: A = green; G = blue.

3.5. *SNaPshot analysis of single breast cancer cells deposited onto AmpliGrid slides*

SNaPshot analysis was also performed after the deposition of single MCF-7 cells onto AmpliGrids via cell sorting. Results gained for the mutational single cell analysis are illustrated in Figure 7.

As expected the heterozygous mutation at position E545K in the *PIK3CA* exon 9 was detected, although peak height was lower than obtained in the aforementioned experiments.

4. Discussion

Up to now, the detection of relevant therapeutic targets in clinical practice is restricted to the primary tumor and success or failure of anti-cancer therapies is only evaluated retrospectively by the absence or presence of metastases after surgery and therapy. Thus, there is an urgent need for biomarkers for real-time personalized monitoring of the efficacy of systemic adjuvant therapy.

Molecular analysis of CTCs, which might reflect certain subpopulations of the primary tumor as well as cells forming metastases, could overcome current limitations. Therefore, we think that SNaPshot analysis of heterogeneous primary tumor samples is not suitable for prediction and that monitoring CTCs as "liquid biopsy" (Pantel et al., 2009) in contrast to analysis of the primary tumor can be utilized to determine the efficacy of chemotherapies and to gain deeper insights into the selection of resistant tumor cells under biological therapies. So far, the CS is the only FDA-approved diagnostic test to automate the detection and enumeration of CTCs, for monitoring disease progression and therapy efficacy in metastatic prostate, colorectal and breast cancer (Cristofanilli et al., 2005). However, besides enumeration, molecular characterization of CTCs is mandatory to not only confirm their malignant origin. Discovering an association between gene expression profiles or expression of gene variants and clinical outcome, and identification of diagnostically and therapeutically relevant targets in CTCs may help stratify cancer patients for individual therapies (Sieuwerts et al., 2009). According to already published data (Fehm et al., 2010), we identified HER2-positive CTCs in the blood samples of patients with HER2-negative primary tumors (Table 1).

Furthermore, by dint of CTC analysis our knowledge of basic molecular pathways of invasion, migration and immune surveillance can be expanded and might contribute to the identification of metastatic stem cells with important implications for the development of improved therapies in the near future (Korkaya and Wicha, 2009). Assessing the presence of target antigens on CTCs could be considered as a real-time biopsy allowing the evaluation of changes in tumour phenotype during the clinical course of the disease. A combination of highly sensitive multi-parametric molecular methods and imaging has been evaluated very recently for the molecular characterization of CTCs (Punnoose et al., 2010). However, this has been hindered by the very limited sample amount available.

In this manuscript we describe the development of an approach to simultaneously determine several SNPs in the *PIK3CA* gene in order to characterize CTCs of metBC patients. This assay combines multiplex PCR amplifications of exons 9 and 20 of the *PIK3CA* gene combined with a multiplex primer extension assay allowing targeted detection of several mutations in one reaction. This so-called SNaPshot technology

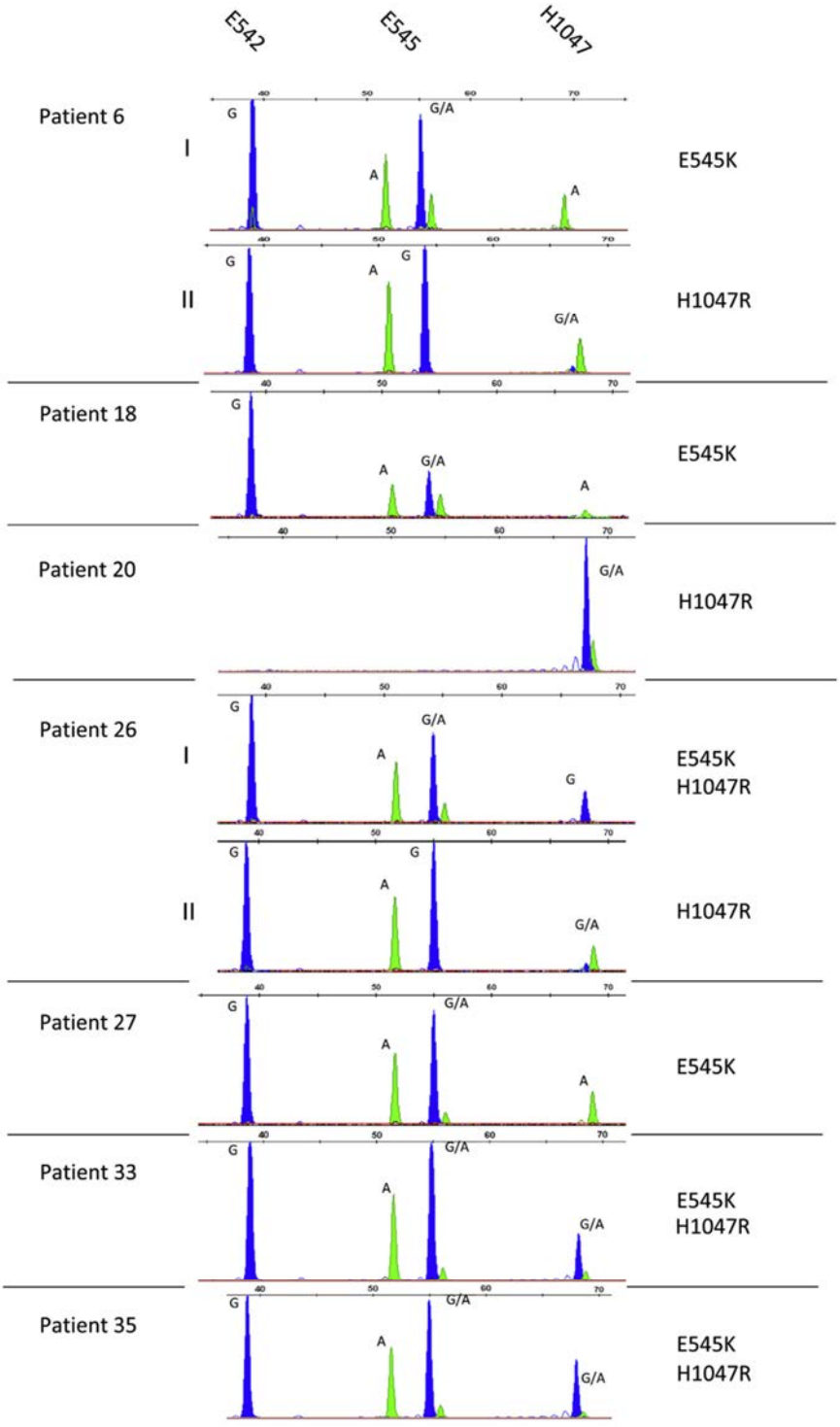

Figure 6 – SNP analysis of hotspot mutations in the *PIK3CA* gene in circulating tumor cells from 7 metBC patients. SNaPshot electropherograms were obtained after CTC enrichment with the Veridex CellSearch® system, Whole Genome Amplification and gene-specific PCRs for exon 9 and exon 20 of the *PIK3CA* gene. For patient (pt) 6 two blood samples were available exhibiting different mutations: sample I showed an E545K mutation while in sample II mutation H1047R was detected. For pt 26 *PIK3CA* mutations E545K and H1047R were observed separately in 2 samples. Pts 18 and 27 revealed mutant alleles at position E545, the sample of pt 20 was merely mutated in exon 20 (H1047R). In CTC samples for pts 33 and 35 mutations E545K and H1047R were observed. Incorporated bases are represented by the following colors: A = green; C = black; G = blue.

Table 3 – *PIK3CA* mutations detected in 7 metastatic breast cancer patients, CTC counts and HER2 status of CTCs within the patients.

Patient	CTC count	Her2 status CTC patient	PIK3CA mutation
6[b]	7; 7	Positive	H1047R; E545K
18	121	Positive	E545K
20	4950	Positive	H1047R
26*	345; 487	Positive	H1047R; E545K/H1047R
27	[a]	Positive	E545K
33	7	Negative	E545K, H1047R
35	2	Positive	E545K, H1047R

a Not determined, because of ferrofluid in CS cartridge; but ≥ 1 CTC determined by visual microscopic inspection.
b 2 different blood samples were analyzed.

has been published by Hurst et al. (2009). However, they applied it to characterize primary cancer tissues. We have modified the original approach to analyse – to our knowledge for the first time – rare CTCs out of breast cancer patients' blood extending it for single breast cancer cells. Regarding tumor heterogeneity and the fact that the correlation of molecular characteristics with outcome helps to identify predictive and prognostic parameters in breast cancer, the method comprising CTC enrichment, WGA and SNaPshot assay represents a novel research approach without being limited by the amount of cells.

Sensitivity tests showed that the SNaPshot technology could detect mutant DNA when it represents 5–10% of the total DNA achieving 100% concordance with results from high-resolution melting analysis and sequencing (Hurst et al., 2009). In our experiments, the concordance rate between SNaPshot and sequencing was 100% as well (Figure 4). In literature, frequency distributions of exon 9 and exon 20 hotspot mutations among *PIK3CA* mutant breast cancer are summarized as 20% for E545K and 55% for H1047R, while E542K and E545G appear less frequent (11% and <1%) (Table 4; adapted from COSMIC, www.mycancergenome.com). For establishing the SNaPshot technology three cell lines were included displaying the wild type *PIK3CA* gene and harboring mutations leading to E545K (MCF-7) and H1047R (T47D), respectively. An additional cell line comprising the E542K

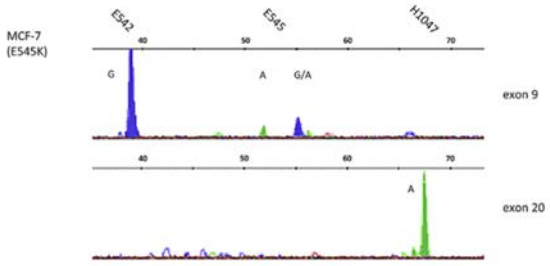

Figure 7 – SNaPshot detection on single MCF-7 breast cancer cells. Top: SNaPshot analysis performed on PCR product for *PIK3CA* exon 9 showing a heterozygous mutation at position E545K; bottom: result for SNP detection in exon 20 of single MCF-7 cells; A = green, G = blue.

was not available in our laboratory, however, several publications described the existence of this mutation in a breast cancer cell line (BT483: Hollestelle et al., 2007; Jensen et al., 2011; Weigelt et al., 2011) and the feasibility of our SNaPshot assay at this position could be proven (wild type pattern -G instead of an A- is displayed).

Although the overall sample number we investigated was very low, we were able to detect mutations in 15.8% (9/57) of the CTC-positive samples, with E545K and H1047R mutations occurring at the same frequency (each 50%, 6/12). Furthermore, occurrence of these somatic mutations seems to be dependent on the breast cancer subtype. For instance, 30% of hormone receptor–positive tumors exhibit *PIK3CA* mutations, while the incidence in triple-negative breast cancer seems to be less frequent (3.3%, Stemke-Hale et al., 2008). Within our collective comprising 7 patients having CTCs with mutations in the *PIK3CA* gene locus, one patient harbored a triple-negative tumor, whereas the other 6 patients had hormone receptor-positive primary tumors (Supplemental data Table 1).

Albeit the impact of these mutations concerning patient outcome is not completely clear yet, it has been reported that PI3K hyperactivity contributes to a lower response to trastuzumab and lapatinib treatment in patients with HER2-positive tumors (Berns et al., 2007; Eichhorn et al., 2008; Serra et al., 2008; Kataoka et al., 2010) as well as to resistance to anti-estrogen therapies. Furthermore, mutations regarding exon 20 of *PIK3CA* have been associated with poor prognosis (Lai et al., 2008). Jensen and co-workers reported that *PIK3CA* gene mutations may be discordant between primary breast cancer and corresponding metastases (Jensen et al., 2011).

CTC population like its primary tumor is heterogenic in its cellular composition in terms of their phenotype and genotype. We observed this heterogeneity in blood samples obtained from different patients as well as in CTCs isolated from the same blood sample (Figure 2). Although we have not yet separated single CTCs in our experiments, we also observed heterogeneity in *PIK3CA* alleles supporting the published data and the need for single cell analysis.

In their recent publication Hurst et al. (2009) state that the identification of mutations using this approach is more straightforward compared to sequencing. In their hands, sequence analysis failed in 17% of the samples. One reason might be as the authors argue that especially in situations of low quality DNA, e.g. from FFPE-tissue samples, the SNaPshot method has the advantage that the fluorescent signals are distributed over fewer peaks. This might also be beneficial regarding CTC analysis, since the CTCs identified using CS have been run through several staining, washing and fixation procedures.

For analysis of all the mutations by sequencing one would have to perform independent PCRs for the 2 exons, followed by 4 bidirectional sequencing reactions. In the SNaPshot assay two multiplex PCRs for exons 9 and 20 are followed by one multiplex detection assay and one capillary electrophoresis run.

One notable limitation of this particular SNaPshot technology might be that the application is limited to a small number of known SNPs. In order to investigate *PIK3CA* mutations which concentrate within only few hot spots this is feasible, since up to 10 SNPs can be screened at once. As aforementioned the sensitivity of the SNaPshot methodology was reported to be

Table 4 – Frequency of exon 9 and exon 20 hot spot mutations among *PIK3CA* mutant breast cancer (modified after www.mycancergenome.com).

Gene	Exon	Location	Amino Acid position	Amino Acid change	Nucleotide change	Mutation frequency
PIK3CA	9	Helical domain	E542	p.E542K	c.1624G > A	11%
			E545	p.E545K	c.1633G > A	20%
				p.E545G	c.1634A > G	<1%
	20	Kinase domain	H1047	p.H1047R	c.3140A > G	55%

approximately 5% (Hurst et al., 2009); an even higher detection rate (up to 0.01%) might be achieved by the recently published BEAMing technology (Beads, Emulsification, Amplification, and Magnetics; Higgins et al., 2012) which is also based on PCR amplification of hot spot regions. However, neither the SNaPshot nor the BEAMing technique allows for the discovery of unknown mutations.

5. Conclusion

Taken together, the herein described assay provides a simple and inexpensive tool to determine variants of key signaling proteins in single cells that could readily be extended to analyze SNPs in other therapeutically relevant genes such as PTEN, ER or EGFR. The SNaPshot assay can be performed in high throughput, is robust and objective making it suitable for use in such a diagnostic setting. Regarding *PIK3CA* analysis, it may be used to further characterize e.g. HER2-positive CTCs which are resistant against anti-HER2 targeted therapy using trastuzumab. As specific inhibitors for *PIK3CA* become available, rapid screening of patient samples for mutations will be essential and the assay may be used to select breast cancer patients benefiting from such a treatment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2013.07.007.

REFERENCES

Araújo, W.M., Vidal, F.C.B., Souza, W., Freitas Junior, J., Souza, W., Morgado-Diaz, J., 2010. PI3K/Akt and GSK-3β prevents in a differential fashion the malignant phenotype of colorectal cancer cells. J. Cancer Res. Clin. Oncol. 136, 1773–1782.

Bachman, K.E., Argani, P., Samuels, Y., Silliman, N., Ptak, J., Szabo, S., et al., 2004. The PIK3CA gene is mutated with high frequency in human breast cancers. Cancer Biol. Ther. 3, 772–775.

Bader, A.G., Kang, S., Vogt, P.K., 2006. Cancer-specific mutations in PIK3CA are oncogenic in vivo. Proc. Natl. Acad. Sci. U S A 3, 1475–1479.

Berns, K., Horlings, H.M., Hennessy, B.T., Madiredjo, M., Hijmans, E.M., Beelen, K., et al., 2007. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell. 12, 395–402.

Board, R.E., Thelwell, N.J., Ravetto, P.F., Little, S., Ranson, M., Dive, C., et al., 2008. Multiplexed assays for detection of mutations in PIK3CA. Clin. Chem. 54, 757–760.

Bozionellou, V., Mavroudis, D., Perraki, M., Papadopoulos, S., Apostolaki, S., Stathopoulos, E., et al., 2004. Trastuzumab administration can effectively target chemotherapy-resistant Cytokeratin-19 messenger RNA-positive tumor cells in the peripheral blood and bone marrow of patients with breast cancer. Clin. Cancer Res. 10, 8185–8194.

Cristofanilli, M., Hayes, D.F., Budd, G.T., Ellis, M.J., Stopeck, A., Reuben, J.M., et al., 2005. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J. Clin. Oncol. 23, 1420–1430.

Dunlap, J., Le, C., Shukla, A., Patterson, J., Presnell, A., Heinrich, M., et al., 2010. Phosphatidylinositol-3-kinase and AKT1 mutations occur early in breast carcinoma. Breast Cancer Res. Treat 120, 409–418.

Eichhorn, P.J.A., Gili, M., Scaltriti, M., Serra, V., Guzman, M., Nijkamp, W., et al., 2008. Phosphatidylinositol 3-Kinase Hyperactivation results in lapatinib resistance that is reversed by the mTOR/Phosphatidylinositol 3-Kinase inhibitor NVP-BEZ235. Cancer Res. 68, 9221–9230.

Fehm, T., Müller, V., Aktas, B., Janni, W., Schneeweiss, A., Stickeler, E., et al., 2010. HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. Breast Cancer Res. Treat. 124, 403–412.

Higgins, M.J., Jelovac, D., Barnathan, E., Blair, B., Slater, S., Powers, P., et al., 2012. Detection of tumor *PIK3CA* status in metastatic breast cancer using peripheral blood. Clin. Cancer Res. 18, 3462–3469.

Hollestelle, A., Elstrodt, F., Nagel, J.H.A., Kallemeijn, W.W., Schutte, M.l., 2007. Phosphatidylinositol-3-OH kinase or RAS pathway mutations in human breast cancer cell lines. Mol. Cancer Res. 5, 195–201.

Hurst, C.D., Zuiverloon, T.C., Hafner, C., Zwarthoff, E.C., Knowles, M.A., 2009. A SNaPshot assay for the rapid and simple detection of four common hotspot codon mutations in the PIK3CA gene. BMC Res. Notes 2, 66.

Ignatiadis, M., Kallergi, G., Ntoulia, M., Perraki, M., Apostolaki, S., Kafousi, M., et al., 2008. Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, Mammaglobin a, and HER2 in early breast cancer. Clin. Cancer Res. 14, 2593–2600.

Ikenoue, T., Kanai, F., Hikiba, Y., Obata, T., Tanaka, Y., Imamura, J., et al., 2005. Functional analysis of PIK3CA gene mutations in human colorectal cancer. Cancer Res. 65, 4562–4567.

Isakoff, S.J., Engelman, J.A., Irie, H.Y., Luo, J., Brachmann, S.M., Pearline, R.V., et al., 2005. Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. Cancer Res. 65, 10992–11000.

Jemal, A., Siegel, R., Xu, J., Ward, E., 2010. Cancer statistics 2010. CA Cancer J. Clin. 60, 277–300.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D., 2011. Global cancer statistics. CA Cancer J. Clin. 61, 69–90.

Jensen, J., Laenkholm, A., Knoop, A., Ewertz, M., Bandaru, R., Liu, W., et al., 2011. PIK3CA mutations may Be discordant between primary and corresponding metastatic disease in breast cancer. Clin. Cancer Res. 17, 667–677.

Kang, S., Bader, A.G., Vogt, P.K., 2005. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. Proc. Natl. Acad. Sci. U S A 102, 802–807.

Kataoka, Y., Mukohara, T., Shimada, H., Saijo, N., Hirai, M., Minami, H., 2010. Association between gain-of-function mutations in PIK3CA and resistance to HER2-targeted agents in HER2-amplified breast cancer cell lines. Ann. Oncol. 21, 255–262.

Korkaya, H., Wicha, M.S., 2009. HER-2, Notch, and breast cancer stem cells: targeting an Axis of Evil. Clin. Cancer Res. 15, 1845–1847.

Lai, Y., Mau, B., Cheng, W., Chen, H., Chiu, H., Tzen, C., 2008. PIK3CA exon 20 mutation is independently associated with a poor prognosis in breast cancer patients. Ann. Surg. Oncol. 15, 1064–1069.

Meng, S., Tripathy, D., Shete, S., Ashfaq, R., Haley, B., Perkins, S., et al., 2004. HER-2 gene amplification can be acquired as breast cancer progresses. Proc. Natl. Acad. Sci. U S A 101, 9393–9398.

Müller, V., Pantel, K., 2009. HER2 as marker for the detection of circulating tumor cells. Breast Cancer Res. Treat 117, 535–537.

Pantel, K., Alix-Panabieres, C., Riethdorf, S., 2009. Cancer micrometastases. Nat. Rev. Clin. Oncol. 6, 339–351.

Paradiso, A., Mangia, A., Azzariti, A., Tommasi, S., 2007. Phosphatidylinositol 3-Kinase in breast cancer: where from here? Clin. Cancer Res. 13, 5988–5990.

Powell, A.A., Talasaz, A.H., Zhang, H., Coram, M.A., Reddy, A., Deng, G., et al., May 7, 2012. Single cell profiling of circulating tumor cells: transcriptional heterogeneity and diversity from breast cancer cell lines [Epub ahead of print]. PLoS One. http://dx.doi.org/10.1371/journal.pone.0033788.

Punnoose, E.A., Atwal, S.K., Spoerke, J.M., Savage, H., Pandita, A., Yeh, R., et al., 2010. Molecular biomarker analyses using circulating tumor cells [Epub ahead of print]. PLoS One. http://dx.doi.org/10.1371/journal.pone.0012517.

Qiu, W., Tong, G.X., Manolidis, S., Close, L.G., Assaad, A.M., Su, G.H., 2008. Novel mutant-enriched sequencing identified high frequency of PIK3CA mutations in pharyngeal cancer. Int. J. Cancer 122, 1189–1194.

Riethdorf, S., Müller, V., Zhang, L., Rau, T., Loibl, S., Komor, M., et al., 2010. Detection and HER2 expression of circulating tumor cells: prospective monitoring in breast cancer patients treated in the Neoadjuvant GeparQuattro trial. Clin. Cancer Res. 16, 2634–2645.

Samuels, Y., Diaz JR., L.A., Schmidt-Kittler, O., Cummins, J.M., DeLong, L., Cheong, I., et al., 2005. Mutant PIK3CA promotes cell growth and invasion of human cancer cells. Cancer Cell. 7, 561–573.

Scher, H.I., Jia, X., Bono, J.S., de, Fleisher, M., Pienta, K.J., Raghavan, D., Heller, G., 2009. Circulating tumour cells as prognostic markers in progressive, castration-resistant prostate cancer: a reanalysis of IMMC38 trial data. Lancet Oncol. 10, 233–239.

Serra, V., Markman, B., Scaltriti, M., Eichhorn, P.J.A., Valero, V., Guzman, M., et al., 2008. NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. Cancer Res. 68, 8022–8030.

Sieuwerts, A., Kraan, J., Bolt-de Vries, J., Spoel, P., Mostert, B., Martens, J.M., et al., 2009. Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. Breast Cancer Res. Treat 118, 455–468.

Simi, L., Pratesi, N., Vignoli, M., Sestini, R., Cianchi, F., Valanzano, R., et al., 2008. High-resolution melting analysis for rapid detection of KRAS, BRAF, and PIK3CA gene mutations in colorectal cancer. Am. J. Clin. Pathol. 130, 247–253.

Stemke-Hale, K., Gonzalez-Angulo, A.M., Lluch, A., Neve, R.M., Kuo, W., Davies, M., et al., 2008. An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. Cancer Res. 68, 6084–6091.

Strati, A., Markou, A., Parisi, C., Politaki, E., Mavroudis, D., Georgoulias, V., Lianidou, E., 2011. Gene expression profile of circulating tumor cells in breast cancer by RT-qPCR. BMC Cancer 11, 422.

Weigelt, B., Warne, P.H., Downward, J., 2011. PIK3CA mutation, but not PTEN loss of function, determines the sensitivity of breast cancer cells to mTOR inhibitory drugs. Oncogene 30, 3222–3233.

Xenidis, N., Perraki, M., Kafousi, M., Apostolaki, S., Bolonaki, I., Stathopoulou, A., et al., 2006. Predictive and prognostic value of peripheral blood Cytokeratin-19 mRNA-positive cells detected by real-time polymerase chain reaction in Node-negative breast cancer patients. J. Clin. Oncol. 24, 3756–3762.

Zhao, J.J., Liu, Z., Wang, L., Shin, E., Loda, M.F., Roberts, T.M., 2005. The oncogenic properties of mutant p110α and p110β phosphatidylinositol 3-kinases in human mammary epithelial cells. Proc. Natl. Acad. Sci. U S A 102, 18443–18448.

3.1.2 EpCAM-independent enrichment of circulating tumor cells in metastatic breast cancer

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RESEARCH ARTICLE

EpCAM-Independent Enrichment of Circulating Tumor Cells in Metastatic Breast Cancer

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Abstract

Circulating tumor cells (CTCs) are the potential precursors of metastatic disease. Most assays established for the enumeration of CTCs so far–including the gold standard CellSearch—rely on the expression of the cell surface marker epithelial cell adhesion molecule (EpCAM). But, these approaches may not detect CTCs that express no/low levels of EpCAM, e.g. by undergoing epithelial-to-mesenchymal transition (EMT). Here we present an enrichment strategy combining different antibodies specific for surface proteins and extracellular matrix (ECM) components to capture an $EpCAM^{low/neg}$ cell line and $EpCAM^{neg}$ CTCs from blood samples of breast cancer patients depleted for EpCAM-positive cells. The expression of respective proteins (Trop2, CD49f, c-Met, CK8, CD44, ADAM8, CD146, TEM8, CD47) was verified by immunofluorescence on $EpCAM^{pos}$ (e.g. MCF7, SKBR3) and $EpCAM^{low/neg}$ (MDA-MB-231) breast cancer cell lines. To test antibodies and ECM proteins (e.g. hyaluronic acid (HA), collagen I, laminin) for capturing $EpCAM^{neg}$ cells, the capture molecules were first spotted in a single- and multi-array format onto aldehyde-coated glass slides. Tumor cell adhesion of $EpCAM^{pos/neg}$ cell lines was then determined and visualized by Coomassie/MitoTracker staining. In consequence, marginal binding of $EpCAM^{low/neg}$ MDA-MB-231 cells to EpCAM-antibodies could be observed. However, efficient adhesion/capturing of $EpCAM^{low/neg}$ cells could be achieved via HA and immobilized antibodies against CD49f and Trop2. Optimal capture conditions were then applied to immunomagnetic beads to detect $EpCAM^{neg}$ CTCs from clinical samples. Captured CTCs were verified/quantified by immunofluorescence staining for anti-pan-Cytokeratin (CK)-FITC/anti-CD45 AF647/DAPI. In total, in 20 out of 29 EpCAM-depleted fractions (69%) from 25 metastatic breast cancer patients additional $EpCAM^{neg}$ CTCs could be identified [range of 1–24 CTCs per sample] applying Trop2, CD49f, c-Met, CK8 and/or HA magnetic enrichment. $EpCAM^{neg}$ dual-positive ($CK^{pos}/CD45^{pos}$) cells could be traced in 28 out of 29 samples [range

1–480]. By single-cell array-based comparative genomic hybridization we were able to demonstrate the malignant nature of one EpCAM^{neg} subpopulation. In conclusion, we established a novel enhanced CTC enrichment strategy to capture EpCAM^{neg} CTCs from clinical blood samples by targeting various cell surface antigens with antibody mixtures and ECM components.

Introduction

CTCs are cancer cells that actively invaded ("motile cells") or have been shed ("mobile cells") from the primary tumor into the blood circulation [1]. Therefore, they are considered as cells with metastatic progenitor characteristics and might be useful surrogates for cancer progression and heterogeneity. Indeed, CTCs have been shown to represent a powerful tool to optimize personalized management of metastatic breast cancer. They are of strong clinical value [2–4] and can be assessed as "liquid biopsy" [5] in a fairly easy, fast, and low invasive fashion. It has been estimated that 1g of tumor tissue (10^9 cells) sheds about 3–4x10^6 tumor cells into the blood stream per day [6]. Most of these cells may be cleared by first-pass effects or die in the hostile environment of the blood [7], which may–among other factors–contribute to the extreme rarity of CTCs within the peripheral blood flow. Consequently, highly sensitive and specific methods for detection, isolation and molecular characterization in the background of supernumerary blood cell components (1 CTC per 10^6–10^7 peripheral mononuclear blood cells) are needed [8–11]. Up to now, various marker-dependent and marker-independent technological advancements have been undertaken for an improved CTC capturing including immunomagnetic, microfluidic and size- as well as function-based methods [12–18]. Marker-dependent approaches using antibodies against EpCAM such as the FDA-approved CellSearch device are dominating recent enrichment strategies. However, considering phenotypic heterogeneity and potential invasion-associated phenotypic plasticity of CTCs, such as epithelial-to-mesenchymal transition (EMT) [19–22] which results in down-regulation of epithelial proteins (including EpCAM), conventional EpCAM-based capturing techniques might miss CTC subpopulations with a more mesenchymal phenotype. Although it has been recently reported that EpCAM-negativity might refer to highly aggressive and invasive CTCs [22, 23], the impact of EMT-like cancer cells on metastatic tumor spread still has to be unraveled. Consequently, to achieve a better understanding of CTC biology in order to overcome treatment failure and to improve disease monitoring/prediction, it is of utmost importance to capture all sorts of CTC subgroups. Thus, to overcome EpCAM-dependence, alternative markers for more comprehensive and efficient CTC detection approaches have to be defined.

Within the presented study, we aimed to improve CTC enrichment/blood testing in an EpCAM-independent manner providing the opportunity to target multiple epithelial- and/or cancer-related antigens expressed on CTCs simultaneously. After intensive literature search we selected several cell surface-specific antibodies (anti-Trop2, -CD49f, -CD146, -CK8, -c-Met, -CD44, -CD47, -AQP5, -ADAM8, -TEM8) [24–30] and components of the extracellular matrix (laminin, collagen I, HA) either immobilized on planar surfaces or coupled to immunomagnetic beads. Efficient cell binding ability was first examined on breast cancer cell lines. In a second step, EpCAM-depleted supernatants comprising potential CTCs that had escaped EpCAM-based selection were used to evaluate our markers in metastatic breast cancer samples. In summary, these fractions were successfully enriched/analyzed for potential EpCAM^{neg}

$CK^{pos}/CD45^{neg}$ events and array-based comparative genomic hybridization of single cells confirmed the malignant origin of one $EpCAM^{neg}$ subpopulation.

Material and Methods

Cell lines and culture conditions

MCF7, SKBR3, HCC1500, ZR-75-1 (all $EpCAM^{pos}$) and MDA-MB-231 ($EpCAM^{low/neg}$) breast cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, US). TMX2-28 cells ($EpCAM^{pos}$) were a generous gift from Prof. K.F. Arcaro (University of Massachusetts, MA, US) [31]. All cell lines were cultured in RPMI 1640 containing 10% (v/v) fetal calf serum and 1% (v/v) penicillin/streptomycin (all Gibco/Life Technologies, Darmstadt, Germany). SKBR3 and ZR-75-1 cells were cultured without any supplements, whereas culture media for MCF7 and TMX2-28 were supplemented with 25 mM HEPES. MDA-MB-231 cells were maintained with supplementation of 2 mM L-glutamine and 20 mM HEPES; for HCC1500 cells 2 mM L-glutamine, 1 mM sodium pyruvate (all Gibco/Life Technologies) and 0.45% (v/v) D-(+) glucose solution (Sigma-Aldrich, Munich, Germany) were added. All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. The same culture conditions were fulfilled for cells grown on coverslips in order to check for marker expression by immunofluorescence staining. At approx. 80% confluence, cells were washed once with PBS (Life Technologies) and fixed with ice cold methanol. Until further use, coverslips were stored at -20°C.

Patient material

Patient samples were gathered within the German DETECT III/IV trials (III: NCT01619111, IV: NCT02035813) wherein patients with primarily HER2-negative metastatic breast cancer are screened for the HER2-status of CTCs (for more information: www.detect-studien.de). Written informed consent was obtained from all participating patients and the study was approved by the Ethical Committee of the Eberhard-Karls University Tuebingen (responsible for DETECT III: 525/2011AMG1) and the local Ethical Committee of the Heinrich-Heine University Duesseldorf (DETECT III: MC-531; DETECT IV: MC-LKP-668). For these patients CellSearch (CS) analysis (Janssen Diagnostics, LLC, Raritan, NJ, US) was routinely performed as described before [32], and additionally, supernatants of EpCAM-depleted fractions were collected. EpCAM-depleted fractions from 25 patients were obtained and processed. Out of these 25, 4 patients donated twice, resulting in a total of 29 analyzed samples.

Search strategy for cell surface markers

Upfront, a literature search was performed in PubMed (http://www.ncbi.nlm.nih.gov/pubmed), from January 2011 to July 2014, for articles evaluating cell surface markers/ECM molecules for CTC enrichment in breast cancer and other tumor entities using the following keywords: 'circulating tumor cells', 'breast cancer', and 'EpCAM negative'. Herein, searches were checked for marker evaluation using commercially available antibodies.

Cytospin preparation

To test for antigen expression, immunofluorescence staining of fixed cells was performed. For this, cell lines were either spun onto glass slides or cultured on coverslips. Cytospins were prepared as follows: cells were harvested into single cell suspension with 1x StemPro Accutase (Life Technologies), transferred into 15 ml tubes and washed once by centrifugation (1000 rpm, RT, 5 minutes) with subsequent pellet resuspension in PBS. The cell count was determined by adding 10 µl cell suspension to a C-Chip Neubauer improved device (PEQLAB

Biotechnologie GmbH, Erlangen, Germany). Afterwards, $5x10^4$ cells/500 μl PBS were spun onto SuperFrost slides (R. Langenbrinck, Emmendingen, Germany) using a ROTOFIX 32 A centrifuge (800 rpm, 2 minutes; Hettich GmbH & Co.KG, Tuttlingen, Germany). Supernatant was removed by aspiration and cytospins were left to dry overnight at RT. Until further use, slides were stored at -20°C.

Antibodies

The following antibodies were used for immunofluorescence staining and immobilization on surface-modified glass slides (NEXTERION, Schott Technical Glass Solutions GmbH, Jena, Germany) and/or on immunomagnetic beads: anti-pan Cytokeratin-FITC (clone C-11) (GeneTex, Irvine, US), anti-EpCAM (clone Ber-EP4) (Dianova GmbH, Hamburg, Germany), anti-EpCAM (clone VU1D9) (Cell Signaling Technology, Cambridge, UK). Anti-Cytokeratin 8 (clone C51), anti-CD45-Alexa Fluor 647 (clone 35-Z6) and anti-ADAM8, rabbit polyclonal (cat. no. sc-25576) were all obtained from Santa Cruz Biotechnology, Dallas, TX, US. Anti-CD49f (clone GoH3), anti-CD146 (clone N1238), anti-TEM8, rabbit polyclonal (cat. no. ab21270) and rat IgG2a, kappa monoclonal (clone RTK2758) were acquired from Abcam, Cambridge, UK. Anti-Trop2 (clone 162–46) and mouse IgG1 isotype control (clone MOPC-21) were purchased from BD Pharmingen, Heidelberg, Germany. Anti-CD47, sheep polyclonal (cat. no. AF4670), anti-HGF R/c-MET (clone 95106) and isotype controls for rabbit (cat. no. AB-105-C) and sheep (cat. no. 5-001-A) IgG were supplied by R&D Systems, Minneapolis, MN, US. Anti-CD44, rabbit polyclonal (cat. no. HPA005785) was from Sigma-Aldrich, Munich, Germany.

For immunofluorescent labeling of cells on cytospins or grown on coverslips, respectively, the following secondary antibodies were used: Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) Antibody, Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) Antibody, Alexa Fluor 594 Goat Anti-Mouse IgG (H+L) Antibody, Alexa Fluor 594 Goat Anti-rabbit IgG (H+L) Antibody, Alexa Fluor 594 Goat Anti-rat IgG (H+L) and Alexa Fluor 488 Donkey Anti-Sheep IgG (H+L) Antibody (all Life Technologies).

Immunofluorescence

Cytospins were thawed and fixed with PBS/4% (v/v) paraformaldehyde for 10 minutes at RT. Slides were then washed three times for 3 minutes with 1x Wash Buffer (Dako, Hamburg, Germany). Cells on cytospins as well as cells grown on coverslips were permeabilized with PBS/0.1% (v/v) Triton-X-100 for 10 minutes on ice and washed three times. In case of secondary antibody application, one drop of protein-block (serum-free; Dako) was applied, incubated for 30 minutes in a humid chamber and decanted. Primary antibody incubation (1:100–1:50) was performed for 1 hour at RT. After three washing steps, cells were incubated with secondary fluorescent antibodies (1:200) together with DAPI (1 μg/ml; Sigma-Aldrich) for 30 minutes. All antibodies were diluted in Antibody Diluent (Dako). Slides/coverslips were washed again and finally mounted with Fluorescence Mounting Medium (Dako). Samples were stored at 4°C until imaging with an Axioplan 2 microscope (Zeiss, Goettingen, Germany).

Generation of single- and multi-marker microarrays

For cell adhesion experiments on planar surfaces, single- and multi-marker microarrays comprising different capturing molecules (antibodies and ECM molecules, see S1 Table and S1 Fig) were produced. Herein, different surface-functionalized NEXTERION coated glass slides (Aminosilane, Epoxysilane, Nitrocellulose, Hydrogel, and Aldehydesilane (AL); Schott Technical Glass Solutions GmbH, Jena, Germany) were available and tested for our application.

Initially, 5x5 spot arrays with 16 arrays per slide were generated (spotting molecules and array layout see S1 Fig). All reagents were diluted in PBS to a final concentration of 0.2 mg/ml and spotted onto the slides, whereas 20 single-droplets of 0.4 nl volume per reagent and spot were deposited using a piezoelectric non-contact print robot (NanoPlotter 2, GeSiM, Grosserkmannsdorf, Germany). The spot diameter was about 500 μm with a spot-to-spot distance (pitch) of 1 mm. After spotting, the slides were blocked with StabilGuard Immunoassay Stabilizer (SurModics, Eden Prairie, MN, US), thoroughly washed with double-distilled water, dried and stored at 4°C in the dark until further use. Optimal antibody concentrations for extended multi-marker array generation were determined. Therefore, purified anti-EpCAM [Ber-EP4], anti-Trop2 and anti-CD49f antibodies were titrated in eight dilutions (0.2, 0.1, 0.05, 0.025, 0.01, 0.005, 0.002, 0.0 mg/ml) in duplicates and spotted manually onto NEXTERION slides AL, resulting in 16 spots per slide and antibody. A single spot consisted of a 1 μl-droplet producing a diameter of approx. 2 mm. Slides were blocked and stored as described above. The final multi-marker array layout consisted of 36 (6x6) spots placed in a total area of 5x5 mm^2. The spot diameter was about 500 μm with a pitch of 800 microns. Therein, anti-CD49f, anti-Trop2, anti-EpCAM, laminin, collagen I and HA spots were immobilized in both, individually and in combination (antibody-antibody, antibody-ECM, ECM-ECM). After printing, the arrays were handled as described above. Immediately, prior to cell incubation, slides were washed three times for 5 minutes with PBS, disposable fluid chambers (custom-designed by STRATEC Biomedical AG, Birkenfeld, Germany) were attached to completely dried slides and arrays were equilibrated with 300 μl RPMI1640/25 mM HEPES for 2 minutes at RT.

Cell adhesion on single- and multi-marker arrays

Cell adhesion experiments on single- and multi-marker arrays were performed with a pool of $EpCAM^{pos}$ cells (MCF7, SKBR3, HCC1500, TMX2-28 and ZR-75-1) in comparison to MDA-MB-231 ($EpCAM^{low/neg}$). $EpCAM^{pos}$ cell lines were pooled in order to assure functionality of applied capture approaches.

Cell adhesion on manually spotted single-marker arrays. Slides with manually spotted antibodies were washed three times with PBS and were then assembled with ProPlate multi-array chambers (16-Square Well; Grace Bio-Labs, Bend, US) according to manufacturer's instructions. Each cavity was filled with $2.5x10^4$ cells (either cell pool or MDA-MB-231) adjusted to 250 μl with RPMI1640/25 mM HEPES and cell adhesion was analyzed after incubation for 2 hours at 37°C and 600 rpm. Supernatants were removed and cavities were washed once with PBS (+$MgCl_2$/$CaCl_2$). Bound cells were visualized by staining with 0.05% w/v Coomassie Brilliant Blue (10 minutes; and 3x5 minutes PBS wash) and subsequent imaging with a Nikon Eclipse TE2000-U microscope (Nikon GmbH, Duesseldorf, Germany).

Cell adhesion on multi-marker microarrays. To discriminate both cell populations ($EpCAM^{pos}$ *vs* $EpCAM^{low/neg}$) after adhesion, $2x10^5$ cells were stained with either 1 μM MitoTracker Green FM (cell pool) or MitoTracker Orange CM (MDA-MB-231) (Molecular Probes/Life Technologies) for 45 minutes at 37°C (protected from light). Afterwards, cells were washed with RPMI1640/25 mM HEPES and 300 μl cell suspension ($EpCAM^{pos}$ plus $EpCAM^{low/neg}$) was applied for array incubation onto prepared NEXTERION AL slides. Incubation of cells lasted for 2 hours at 37°C with horizontal shaking (450 rpm). After incubation, disposables were rinsed twice, then removed and slides were mounted with 20 μl VECTASHIELD Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA, US) and were finally covered for imaging with a BIOREVO BZ-9000 fluorescence microscope (KEYENCE, Neu-Isenburg, Germany). Total cell fluorescence was quantified with the ImageJ/Fiji 1.46 software [33].

Immunomagnetic bead coating and cell capture

Positive selection of breast cancer cell lines as well as of potential tumor cells within the EpCAM-depleted sample fractions was achieved by employing immunomagnetic enrichment with either Dynabeads (Life Technologies) or Bio-Adembeads (Ademtech, Pessac, France) coated with antibodies or HA. Dynal MPC-S/MPC-L (Life Technologies) magnets were used for magnetic separation of beads.

Direct coating of Dynabeads and Bio-Adembeads with antibodies. Antibodies were coupled to Dynabeads goat anti-mouse IgG, Dynabeads sheep anti-rat IgG and Dynabeads M-280 sheep anti-rabbit IgG according to the manufacturer's protocol. Briefly, after pre-washing the beads with 1 ml PBS/2 mM EDTA/1% (v/v) FCS (isolation buffer), 25 μl (1–1.75x10^7) beads/reaction were incubated with 0.5 μg primary capture antibody for 45 minutes at 4°C while gently tilting and rotating. Afterwards, coated Dynabeads were washed twice in 1 ml isolation buffer, resuspended in the initial buffer volume and stored at 4°C until further use. Additionally, Bio-Adembeads goat anti-mouse IgG and Bio-Adembeads goat anti-rat IgG were used. Coupling conditions were adjusted to those for Dynabeads with incubation, buffer and storage conditions being in accordance with the aforementioned procedure.

Direct coating of Dynabeads with hyaluronic acid. Hyaluronic acid (HA, from *Streptococcus pyrogenes*, Calbiochem/Merck Millipore, Darmstadt, Germany) was coupled to Dynabeads MyOne Carboxylic Acid (Life Technologies). Therefore, 3 mg beads were used for coating with 150 μg HA. HA concentration was adjusted to 2.5 mg/ml in 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; Sigma-Aldrich) buffer, pH 6. Beads were washed twice with 300 μl 25 mM MES, pH 6 for 10 minutes whilst rotating. For bead activation 50 μl 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC; Thermo Fisher Scientific, Rockford, US) and 50 μl *N*-hydroxysulfosuccinimide (sulfo-NHS; Thermo Scientific), diluted in 25 mM MES (pH 6) to a concentration of 50 mg/ml, were added and incubated for 30 minutes at RT and 650 rpm. Supernatant was discarded and beads were washed twice as described before. For ligand immobilization, HA was added to the activated beads in a total volume of 100 μl and after mixing thoroughly, incubated for 30 minutes at RT and 450 rpm. Supernatant was removed and the beads were incubated with 300 μl 50 mM Tris, pH 7.4 (Sigma-Aldrich) for 15 minutes at RT to quench the non-reacted activated carboxylic acid groups. Beads were washed four times with 50 mM Tris, pH 7.4 and were finally resuspended in PBS/0.02% (w/v) NaN_3 to their original volume. Functionalized beads were stored at 4°C until further use. Prior to sample incubation, beads were washed twice with isolation buffer.

Cell capture with Dyna/Adembeads. For each capture reaction, 25 μl (1x10^7)/15 μl coated Dyna/Adembeads were incubated with 2.5x10^5 cells for 30 minutes at RT and tilt rotation. Bead fractions were washed three times, resuspended in 500 μl PBS and transferred to 1.5 ml Protein LoBind tubes (Eppendorf AG, Hamburg, Germany). For imaging, bead/cell suspensions were mounted with 20 μl VECTASHIELD Mounting Medium/DAPI onto microscope slides.

Processing of EpCAM-depleted breast cancer clinical samples and detection of recovered cells

To enrich potential CTCs that had not been captured by the EpCAM-positive selection through the CS system, EpCAM-depleted fractions were further analyzed via immunomagnetic beads coated with specific cancer-related markers. All fractions were preserved by the addition of 300 μl 0.1236 M EDTA for overnight storage at 4°C. For each collected sample fraction (21–39 ml), 2 to 6 separate enrichment approaches were carried out. Whole fraction volumes were equally separated and transferred to 15 ml-tubes. Isolation buffer was added to a final volume

of 10 ml. Then samples were centrifuged (600xg, 10 minutes, RT) and the buffer/plasma fraction was removed. Prior to bead incubation, the remaining white/red blood cell fraction was recompleted to 5 ml. For incubation with single bead preparations (antibody or ECM) 25 μl ($1x10^7$)/15 μl pre-coated Dyna/Adembeads were added, for combined Dynabead approaches (antibody plus antibody/ECM) 12.5 μl each were mixed. Positive cell isolation was obtained as described above. Previous to staining of captured cells, bead/cell suspensions were pre-incubated with 5 μl Human TruStain FcX (Biolegend, San Diego, US) for 5 minutes. Samples were then stained with anti-pan-CK-FITC (1:400), anti-CD45-Alexa Fluor 647 (1:20) and DAPI (1 μg/ml) adjusted to 100 μl with PBS/0.1% Tween 20 for 30 minutes at 4°C and 450 rpm. Beads were washed twice in PBS, deposited onto microscope slides and mounted in Fluorescence Mounting Medium (Dako). Slides were stored at 4°C until imaging with an Axioplan 2 fluorescence microscope (Zeiss, Goettingen, Germany) considering CK^{pos}/$CD45^{neg}$/$DAPI^{pos}$ stain criteria.

Isolation of single cells and molecular characterization of CTCs

Single cell isolation. Single cells were isolated via the CellCelector (CC) device (ALS, Jena, Germany), an automated robotic micromanipulator system, to perform subsequent genomic analysis. Bead-enriched and stained samples from the EpCAM-depleted fractions were screened for CK^{pos}/$CD45^{neg}$ events with the CC, whereas $EpCAM^{Pos}$ CTCs were selected and identified via CS prior to re-identification with the CC. For improved re-identification of $EpCAM^{pos}$ CTCs with the CC, cartridges were re-inserted into the MAGNEST holder, incubated for 15 minutes, and supernatants (approx. 320 μl) were discarded before the staining solution (DAPI (1 μg/ml), anti-CD45-Alexa Fluor 647 (1:20) in 300 μl PBS) was added. Cells were re-stained for 4 hours at 4°C in the dark. After magnetic incubation (15 minutes), the staining solution was removed and cells were washed with 300 μl PBS. Cartridge contents were mixed thoroughly ensuring that all cells dislodged from the cartridge wall, and were transferred onto glass slides for CC analysis. All samples (CS and bead-enriched) were screened for a positive DAPI/CK and a negative CD45 stain and single cells were deposited within 10 μl-droplets of PBS into 0.15-ml PCR tubes. Tubes were centrifuged for 10 minutes at 1300 rpm and 9 μl of PBS were removed. Finally, cells were frozen at -80°C until further processing.

Whole genome amplification. Whole genomes of isolated single cells were amplified using the Ampli1 whole genome amplification (WGA) Kit (Silicon Biosystems, Bologna, Italy) according to the manufacturer's instructions. Herein, the one-day protocol with an extended overnight step for cell lysis was performed. The quality of the Ampli1 WGA output product was evaluated by running the Ampli1 QC Kit (Silicon Biosystems), which assays a multiplex PCR of four markers. Two μl of the WGA product were analyzed according to the manufacturer's protocol. Samples displaying three or four bands in the multiplex PCR were used for array-based comparative genomic hybridization (aCGH) analysis [34].

Array-based comparative genomic hybridization and data analysis. aCGH (4×180k arrays, Agilent Technologies, Waldbronn, Germany) was performed as previously described [34–36] using 1 μg of the primary amplification product. As a reference we used WGA products of single GM14667 cells, a cell line derived from normal male B-lymphocytes. For data analysis the output image files were imported, normalized and the fluorescent ratios for each probe were determined using the Feature Extraction software (Agilent Technologies, Version 10.7.3.1, Protocol CGH_1105_Oct09). Data visualization and analysis was performed with the Genomic Workbench 6.5.0.18 software using the ADM-2 algorithm with a threshold of 6.0, centralization by legacy with a threshold of 4.0 and a bin size of 10.

Results

Evaluation and expression of capture cell surface proteins

To successfully capture EpCAM-negative or -low breast cancer cells, different antibodies specific for cell surface proteins (Trop2, CD49f, CD146, CK8, c-Met, CD44, CD47, AQP5, ADAM8, TEM8) as well as ECM molecules (laminin, collagen I, HA) were tested on breast cancer cell lines with different EpCAM expression levels. These proteins were selected upon literature-based research and were partly described previously with regard to tumor cell enrichment [24–30].

All studied proteins were expressed heterogeneously and to varying degrees in the examined EpCAM^{pos} (MCF7/SKBR3) and EpCAM$^{low/neg}$ (MDA-MB-231) cell lines (Fig 1), with CD49f and CD146 showing the most abundant expression in the EpCAM$^{low/neg}$/basal-like cell line MDA-MB-231. Regarding the expression of Trop2, CD49f as well as ADAM8 within the EpCAM^{pos} cell lines, HER2-amplified SKBR3 cells revealed more abundant expression levels compared to MCF7 (luminal A subtype). Furthermore, protein distribution throughout the cells for CK8 and CD146 differed greatly. For instance, signals for CD146 exhibited membranous and cytoplasmic localization within MDA-MB-231 cells, whereas it was expressed almost exclusively in the nucleus within luminal B subtype cells (e.g. HCC1500, data not shown).

Cell enrichment using single-/multi-marker arrays and immunomagnetic beads

Based on previous tests of various functional substrates on NEXTERION coated glass slides, the AL coating was chosen since it provided the most specific binding of cells on capture spots and low background (S1 Fig). In subsequent titration experiments, the optimal spotting

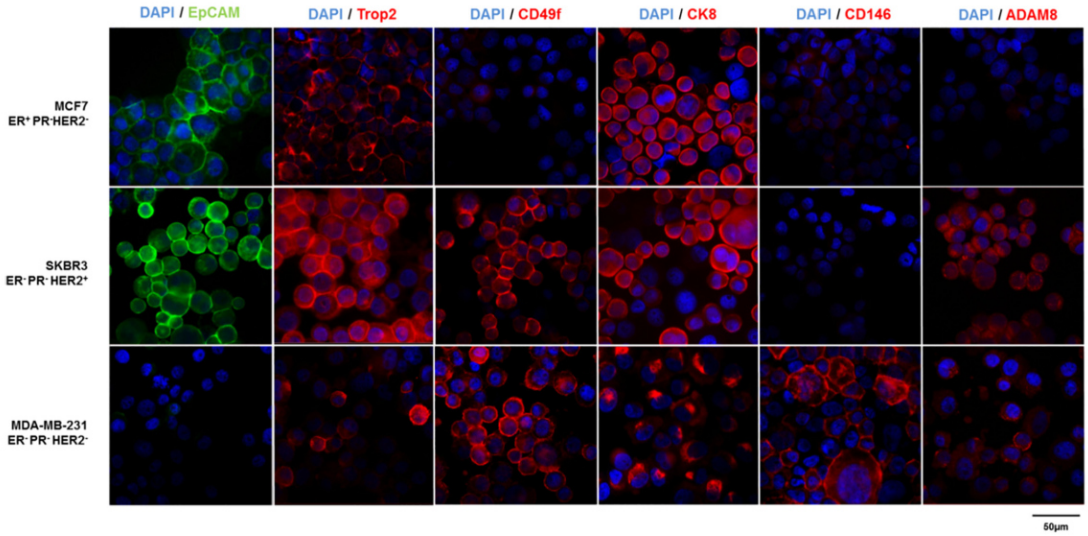

Fig 1. Expression of surface markers on EpCAM^{pos} and EpCAM$^{low/neg}$ cells. Differential protein expression of EpCAM (green), Trop2, CD49f, CK8, CD146 and ADAM8 (red) in EpCAM^{pos} (MCF7, ER$^+$PR$^-$HER2$^-$, top; and SKBR3, ER$^-$PR$^-$HER2$^+$, middle) and EpCAM$^{low/neg}$ (MDA-MB-231, ER$^-$PR$^-$HER2$^-$, bottom) cell lines is displayed by immunofluorescence staining of cytospins; blue = DAPI, 40x magnification.

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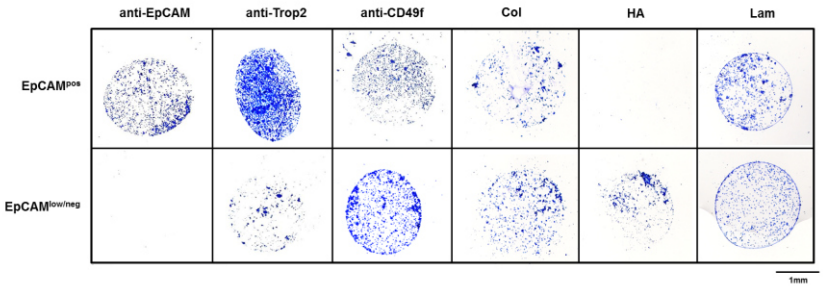

Fig 2. Adhesion of EpCAM$^{pos/neg}$ cells on manually spotted arrays. 2.5x10^4 EpCAM^{pos} cells (pool of MCF7, SKBR3, HCC1500 and ZR-75-1; upper panel) and EpCAM$^{low/neg}$ cells (MDA-MB-231; lower panel) were incubated for cell adhesion experiments on glass substrates (NEXTERION slides AL) manually coated with anti-EpCAM [Ber-EP4], anti-Trop2, anti-CD49f (0.1 mg/ml each), collagen I (Col), hyaluronic acid (HA) and laminin (Lam) (0.2 mg/ml each). Cell adhesion was visualized by Coomassie; 20x magnification.

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concentration of antibodies/ECM molecules was reduced to 0.1 mg/ml leading to maximum cell adhesion as visualized by a positive Coomassie stain. Nonspecific binding to the AL substrate itself (pure spotting buffer) as well as to an isotype control (mouse) or bovine serum albumin could not be observed at any time on single marker arrays (S1 Fig). In case of absent cell binding despite a positive immunofluorescence staining on cytospins—observed for AQP5, CK8 and CD146—successful antibody coupling to the substrate was examined after visualization with AlexaFluor 594. Staining of spotted arrays revealed that coupling with anti-CD146 was unsuccessful, whereas anti-CK8 and anti-AQP5 antibodies bound to the slides, but were non-functional. Examples for successful cell adhesion of EpCAM^{pos} (cell pool of MCF7, SKBR3, HCC1500 and ZR-75-1) and EpCAM$^{low/neg}$ (MDA-MB-231) cells to anti-EpCAM, anti-Trop2, anti-CD49f and three ECM molecules (collagen I, HA, laminin) are representatively shown in Fig 2.

Except for HA, all investigated molecules could capture the EpCAM^{pos} cell pool. According to the Coomassie blue signal, cell adhesion to Trop2 and EpCAM spots was most abundant, however binding to CD49f and collagen/laminin could also be observed. In contrast, MDA-MB-231 cells adhered to Trop2, CD49f and all three ECM spots, while the EpCAM spot did not reveal any cell attachment; and capturing through anti-CD49f and HA, MDA-MB-231 binding was much more prominent compared to the EpCAM^{pos} pool.

In continuation of the initial cell adhesion experiments, single arrays were then extended to 36-spot arrays including further antibodies (CD44, CD47, c-Met, TEM8), isotype controls and marker combinations (ab/ab; ab/ECM; ECM/ECM) for anti-CD49f, anti-Trop2, anti-EpCAM, laminin, collagen I, and HA. Spotted capture molecules are listed in S1 Table.

To distinguish and quantify bound EpCAM$^{pos/neg}$ populations, cells were pre-stained with either MitoTracker green (EpCAM^{pos}) or MitoTracker orange (EpCAM^{neg}) (Fig 3). Total cell fluorescence (integrated density) for each single spot was quantified using the ImageJ/Fiji 1.46 software. Herein, fluorescence signals per spot are displayed as mean values of three individual experiments (S2 Fig). EpCAM^{pos} cells adhered to all spots containing EpCAM antibody (Fig 3A, 3C and 3D: spots 10, 16–21; S2 Fig). Moreover, binding to anti-EpCAM was increased when it was combined with an ECM molecule or anti-Trop2 or anti-CD49f antibodies. Less binding was observed to spots comprising anti-Trop2 (spots 6–9), CD49f (spots 12–15) and CD47 (spot 25) and almost no adhesion to ECM-alone and isotype control (spots 1, 5, 24, 30) spots was obtained.

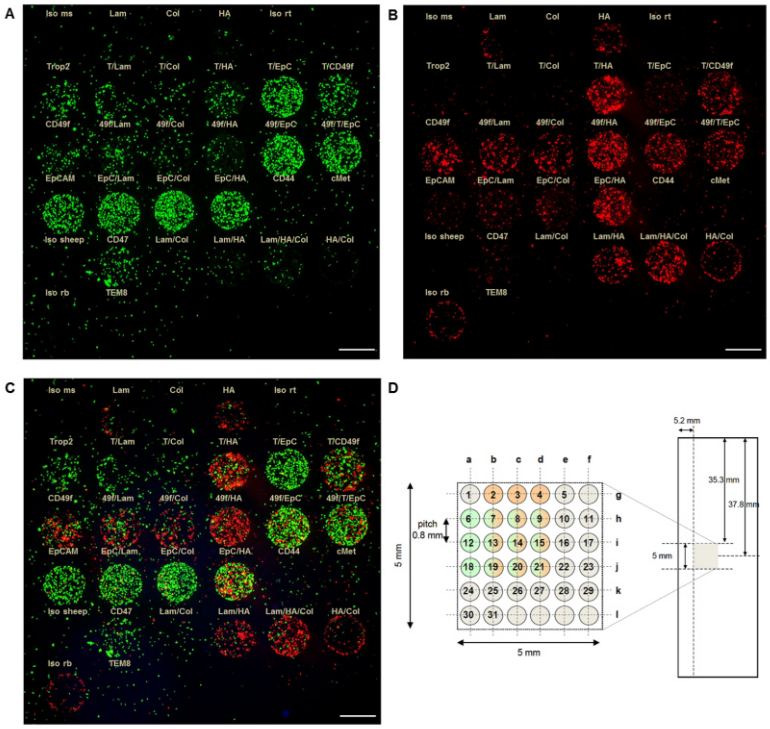

Fig 3. Adhesion of EpCAM$^{pos/neg}$ cells on multi-marker arrays. 2x10^5 EpCAM^{pos} (cell pool of MCF7, SKBR3, HCC1500, ZR-75-1, TMX2-28) (A) and EpCAM$^{low/neg}$ cells (MDA-MB-231) (B) were either stained with 1 μM MitoTracker Green FM (A) or MitoTracker Orange CM (B) and incubated for cell adhesion experiments on NEXTERION slides AL, coated with different antibodies and ECM molecules, alone and in combination (0.1 mg/ml each). The labeling above the single spots indicates respective capture molecules (Iso = isotype control, ms = mouse, Lam = laminin, Col = collagen, HA = hyaluronic acid, rt = rat, T = Trop2, EpC = EpCAM, 49f = CD49f, rb = rabbit). (C) Overlay image of (A) and (B); scale bars (white) = 500 μm, 20x magnification. (D) Array layout (5x5 mm) with 36 spots (spot diameter = 500 μm; pitch = 800 μm) printed on NEXTERION slides AL.

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In contrast, binding of EpCAM$^{low/neg}$ MDA-MB-231 cells revealed a different pattern (Fig 3B–3D and S2 Fig): strong adhesion to anti-CD49f and HA could be observed—both alone (spots 4, 12) and in combination with laminin and collagen (spots 9, 11, 13–17, 21, 27–29). However, only marginal binding for Trop2 and EpCAM spots (spots 6–8, 10, 18–20) was detectable. A slight signal deriving from cells bound to the rabbit isotype control (spot 30) could be identified. Cell adhesion for both populations to spots coated with CD44 (spot 22), c-Met (spot 23) and TEM8 (spot 31) antibodies was almost undetectable (Fig 3C).

After successful testing, surface enrichment protocols in single- and multi-arrayed formats were changed to immunomagnetic bead-based enrichment in order to establish an approach more suitable for clinical samples—first using Dynabeads and later including Adembeads. Adembeads were more compatible with subsequent microscopic analysis (e.g. lower autofluorescence) and cells could easily be distinguished from the beads.

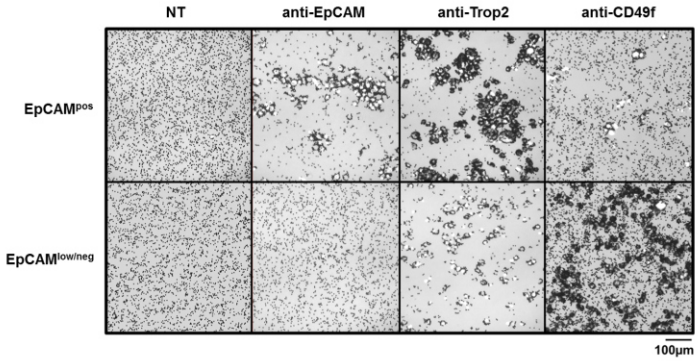

Fig 4. Enrichment of EpCAM$^{pos/neg}$ cells with Dynabeads. EpCAM^{pos} (upper panel; MCF7, SKBR3, T47D, HCC1500 and ZR-75-1) and EpCAM$^{low/neg}$ (lower panel; MDA-MB-231) cells were captured with Dynabeads coupled with antibodies for EpCAM, Trop2 and CD49f (1 µg ab/2.5x10^5 cells/1x10^7 beads). No cells bound to uncoated Dynabeads (NT = no target). Cells were imaged after a DAPI stain and are depicted as brightfield/DAPI merge; 10x magnification.

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All IgG-coated Dyna-/Adembead preparations, both uncoupled and coupled to antibodies, were tested for their cell binding ability. Representative capturing upon coupling to anti-EpCAM, anti-Trop2 and anti-CD49f of pooled EpCAM^{pos} as well as EpCAM$^{low/neg}$ cells is displayed in Fig 4. EpCAM^{pos} cells (Fig 4, upper panel) can be recovered by anti-EpCAM and anti-Trop2 bead preparations and, to a lesser extent by anti-CD49f. MDA-MB-231 cells (Fig 4, lower panel) can be efficiently enriched with beads coupled to anti-CD49f. Besides that, non-specific binding of both cell populations to no-target bead controls could not be detected.

Enrichment of CTCs from EpCAM-depleted breast cancer clinical samples

Blood samples from 25 breast cancer patients (Table 1) were analyzed by CellSearch for EpCAM^{pos} CTCs: 11 out of 29 blood samples from 25 patients were negative for CTCs (8x) or CTC count could not be determined (3x); three samples had 1 CTC and 15 samples harbored at least 3 CTCs (mean: 20, median: 3; range 0–206 CTCs) (Fig 5A; Table 1). Additionally, to apply the established workflow, respective EpCAM-depleted fractions for all samples were collected and afterwards processed with beads coupled to antibodies against either Trop2, CD49f, CK8, CD44, ADAM8, CD146, c-Met or to HA, alone or in combination. The entire volume (21–39 ml), was fractionated and subjected to analysis providing 2 to 6 different enrichments per EpCAM-depleted sample (detailed information see S2 Table) depending on the volume. In total, 96 fractions (65x Dynabeads, 31x Adembeads) were investigated and captured cells were mounted onto glass slides. Total numbers of potential CTCs and double positive cells (Fig 5; Table 1) reflect the sum of all immunomagnetic enrichments for each blood/patient sample. Taken together, 95 potential EpCAM^{neg} CTCs (CK^{pos}/CD45^{neg}; mean/median: 3/2; range 1–24 CTCs) (Figs 5 and 6A), and 1069 dual-positive cells (CK^{pos}/CD45^{pos}; mean/median; 38/9; range 1–480) (Figs 5B and 6B) were identified (Table 2).

Potential EpCAM^{neg} CTCs could be identified in 20 out of 29 EpCAM-depleted fractions (69%) from 25 metastatic breast cancer patients. Moreover, 64% (7 out of 11) of the samples with negative or undetermined EpCAM^{pos} CTC-status revealed at least one EpCAM^{neg} CK^{pos}/

Table 1. Patient characteristics for CTCs/primary tumors and number of $CK^{pos}/CD45^{neg}$ and $CK^{pos}/CD45^{pos}$ events within the EpCAM-depleted fractions.

			CTC		primary tumor				EpCAM-depleted	
#	Pat DIII_DIV	age	CS	HER2 status	ER	PR	HER2 neu	subtype	$CK^{pos}/ CD45^{neg}$	$CK^{pos}/ CD45^{pos}$
1	DIII-1	45	1	neg	pos	pos	neg	lum A	1	15
2	DIII-2	54	0		[b]	[b]	[b]	[b]	24	18
3	DIII-3	71	147	pos	pos	pos	neg	lum A	2	11
4	DIII-4	74	0		pos	pos	neg	lum A	1	2
5	DIII-5	69	13	neg	pos	neg	neg	lum A	2	5
6	DIII-6	76	6	neg	pos	pos	neg	lum A	2	18
7	DIII-7	48	3	neg	pos	pos	neg	lum A	0	100
8	DIII-8	63	206	pos	pos	pos	neg	lum A	17	54
9	DIII-9	70	[a]/[a]		[b]	[b]	[b]	[b]	0/0	2/1
10	DIII-10	45	1	neg	pos	pos	neg	lum A	2	6
11	DIII-11	72	6	pos	pos	neg	neg	lum A	0	1
12	DIII-12	45	1/0		neg	neg	neg	basal	0/0	0/192
13	DIII-13	62	0		pos	pos	neg	lum A	3	480
14	DIII-14	58	12	pos	pos	pos	neg	lum A	0	48
15	DIII-15	38	0		pos	pos	neg	lum A	0	14
16	DIII-16	63	0		pos	pos	neg	lum A	2	13
17	DIII-17	49	[a]/[a]		neg	neg	neg	basal	3/6	2/16
18	DIII-18	75	50	neg	pos	neg	neg	lum A	8	9
19	DIII-19	54	0		pos	pos	neg	lum A	3	3
20	DIV-1	55	10	neg	pos	pos	neg	lum A	7	16
21	DIV-2	70	37	neg	pos	pos	neg	lum A	2	8
22	DIV-3	50	16	neg	pos	pos	neg	lum A	3	9
23	DIV-4	56	3	neg	pos	pos	neg	lum A	0	34
24	DIV-5	66	3	neg	pos	pos	neg	lum A	3	9
25	DIV-6	48	5/3	pos	pos	pos	neg	lum A	3/1	9/4

[a] CTC count could not be determined

[b] unknown

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$CD45^{neg}$ event. In 5 fractions with respective $EpCAM^{pos}$ CTCs [1 to 12 CTCs], no further potential $EpCAM^{neg}$ CTCs could be detected. Regarding $EpCAM^{neg}$ $CK^{pos}/CD45^{pos}$ events, only one sample (#12) did not exhibit any double positive cells, all other samples harbored at least 1 event. A high proportion of $EpCAM^{neg}$ $CK^{pos}/CD45^{pos}$ cells was found in supernatants with corresponding low $EpCAM^{pos}$ CTC numbers in CS (except for patient #8).

Most of the samples were enriched with antibodies against Trop2, CD49f and CK8 (63%) yielding 80% of all identified potential CTCs. All gathered CTC numbers ($EpCAM^{pos/neg}$) as well as double positive cells can be found summarized and linked to patient characteristics in Table 1. Patients eligible for DIII/IV studies suffered from HER2-negative metastatic breast cancer, whereas for two patients (#2, #9) primary tumor characteristics could not be determined. Two patients (#12, #17) harbored triple-negative tumors ($ER^-PR^-HER2^-$); the remaining cohort evinced primary tumors of luminal A subtype (18/21: $ER^+PR^+HER2^-$, 3/21: $ER^+PR^-HER2^-$).

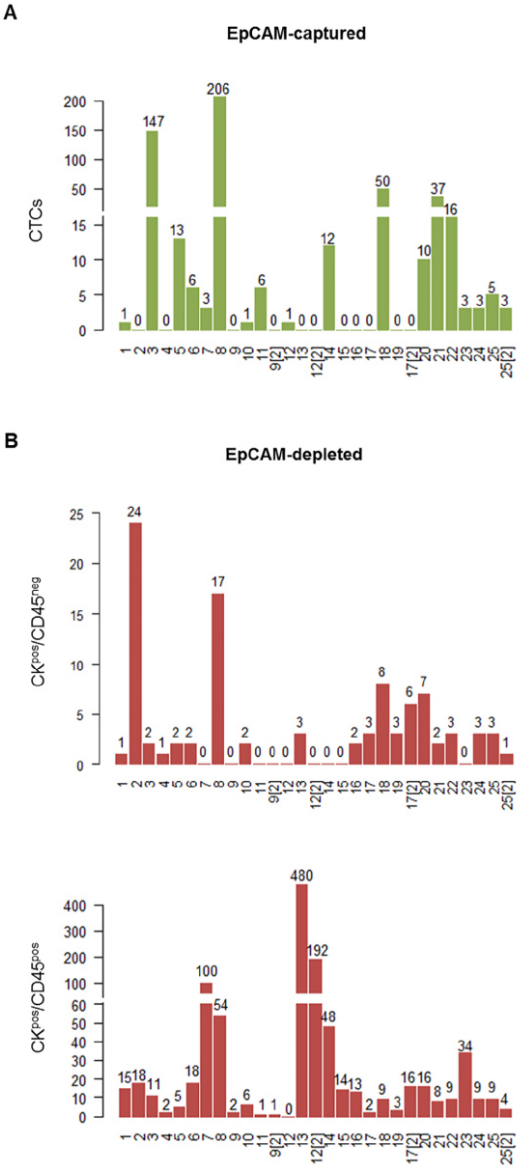

Fig 5. Number of EpCAM-enriched CTCs and potential CTCs/double positive events ($CK^{pos}/CD45^{pos}$) within the EpCAM-depleted fractions in blood of breast cancer patients. (A) CTC count determined by EpCAM-enrichment and subsequent CK/DAPI stain in 29 blood samples of 25 patients (DIII and DIV). (B) Number of potential CTCs (top) and double positive events (bottom) within the respective EpCAM-depleted supernatants of the same blood samples; total event numbers ($CK^{pos}/CD45^{neg}$ and $CK^{pos}/CD45^{pos}$) represent the sum of all events identified after 2–6 immunomagnetic enrichments for each blood/patient sample.

doi:10.1371/journal.pone.0144535.g005

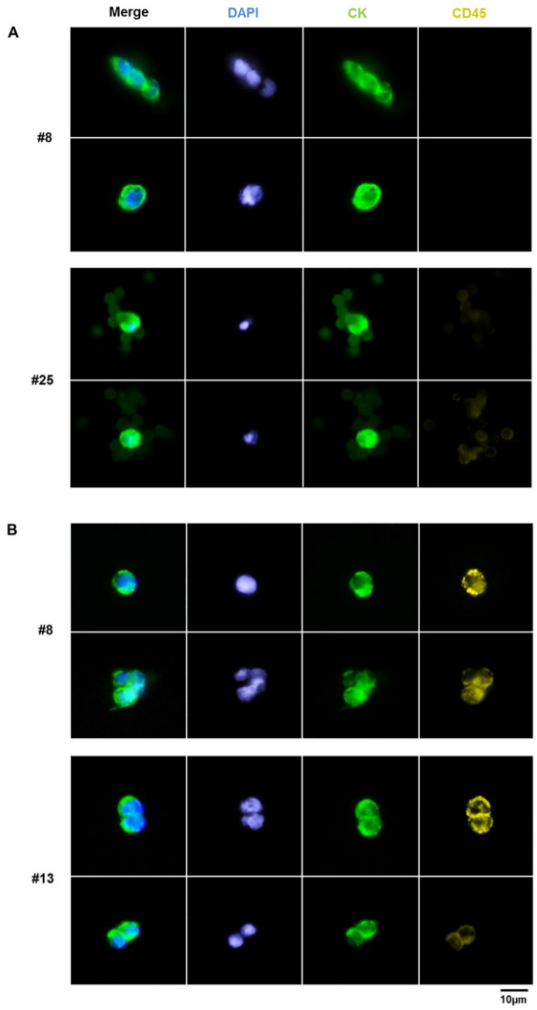

Fig 6. Representative images of potential CTCs ($CK^{pos}/CD45^{neg}$) and double positive ($CK^{pos}/CD45^{pos}$) events enriched from EpCAM-depleted patient samples. Immunofluorescence staining of (A) CTCs (top: #8, Adem-c-Met; bottom: #25, Dyna-CD49f) and (B) $CK^{pos}CD45^{pos}$ events (top: #8, Adem-c-Met; bottom: #13, Adem-Trop2) enriched after EpCAM-depletion are shown. Adem-/Dynabeads captured cells were stained for DAPI (blue), pan-CK (green) and CD45 (yellow); 40x magnification.

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Molecular characterization of isolated single cells

In order to validate that the isolated marker-positive cells (Fig 7A) were indeed cancer cells, we analyzed three CD44-captured $EpCAM^{neg}$ cells in comparison to one $EpCAM^{pos}$ CTC from the same patient for their somatic copy number profiles (Fig 7B). All four cells displayed

Table 2. Number of processed fractions and EpCAM^{neg} CK^{pos}/CD45^{neg} and CK^{pos}/CD45^{pos} events for each marker.

	Processed fractions	CK^{pos}/ CD45^{neg}	CK^{pos}/ CD45^{pos}
Trop2	23	27	210
CD49f	20	22	210
CK8	14	13	97
cMet	27	27	452
ADAM8	11	1	38
CD146	3	3	67
CD44	4	3	8
Hyaluronic acid	9	1	59
total	96	95	1069

Listed are the numbers of processed fractions (in total 96) for each marker as well as EpCAM^{neg} CK^{pos}/CD45^{neg} and CK^{pos}/CD45^{pos} events. The total number of processed samples does not include marker combinations.

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aberrant genomes as expected for tumor cells. We found similar moving averages on chromosome 1, 2 and 11 for both EpCAM^{neg} cells and the EpCAM^{pos} CTC (Fig 7B, highlighted in grey). Besides that, EpCAM^{neg} cells showed a gain of chromosome 5 as well as a deletion at chromosome 8p (Fig 7B, highlighted in blue).

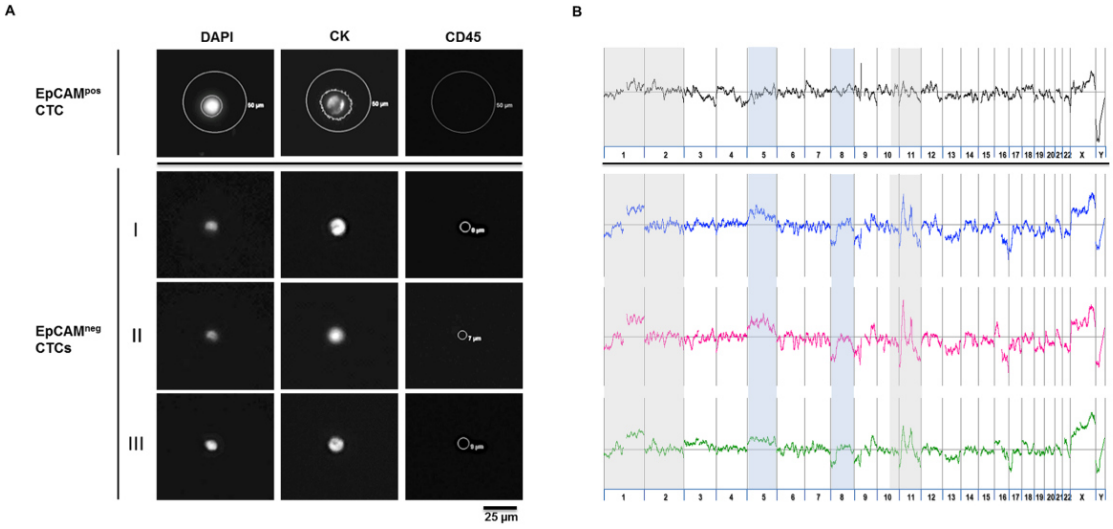

Fig 7. Genomic profiling of CellCelector identified and isolated whole genome amplified EpCAM^{neg} single cells confirms their malignant nature. (A) One EpCAM^{pos} CTC was selected and identified via CellSearch and re-identified with the CellCelector for a positive DAPI and CK-PE (displayed in the TRITC channel), and a negative CD45 (Cy5 channel) stain. Three EpCAM^{neg} CTCs (I, II, III) from the CellSearch EpCAM-depleted fraction of the same patient were enriched with CD44-Adembeads and stained for DAPI/pan-CK-FITC/CD45-AF647. Single cells were isolated via the CellCelector, the whole genomic material was amplified and (B) genome wide aCGH profiles were obtained confirming that EpCAM-independent enrichment captures malignant cells. Chromosomal regions highlighted in grey show common somatic copy number alterations, light blue areas represent different chromosomal aberrations between both CTC populations.

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Discussion

Until now, treatment decisions in breast cancer have been based on the expression profiles of the primary tumors. However, CTCs have been shown to display different molecular features regarding hormone receptor/EGFR and HER2 expression and thereby may elude primary treatment [37–42]. Consequently, optimized prognostic and predictive tests are required to focus on the analysis of disseminated/circulating tumor cells, which represent the actual targets of adjuvant therapies. Several methodologies for CTC enrichment have been developed that rely on EpCAM expression on CTCs. It is known that EpCAM is frequently expressed throughout different tumor entities [43, 44]. However, the loss of the EpCAM antigen has been intensively delineated [45–49]: while its up-/down-regulation has been linked to poor overall survival [50–53], its down-regulation has been described as a part of the EMT process accompanied by an enhanced migratory and metastatic potential [49, 54–56]. Thus, in order to warrant a comprehensive and systematic detection of all epithelial- and mesenchymal-like CTC subpopulations including intermediate states, the EpCAM-based enrichment and isolation techniques (e.g. by CellSearch), as the current gold standard, ought to be improved.

Here we established an integrated workflow, which captures EpCAM-depleted and hence previously unrecognized CTC subsets by using antibodies specific for cell surface proteins (Trop2, CD49f, c-Met, CK8, ADAM8, TEM8, CD44, CD47, CD146) as well as ECM components (laminin, collagen, HA).

As expected, only marginal to absent binding of MDA-MB-231 cells to EpCAM-antibodies on planar surfaces as well as coupled to beads could be observed. In contrast, efficient adhesion/capturing of $EpCAM^{low/neg}$ cells (cell line and/or EpCAM-depleted blood fractions) could be achieved by antibodies specific for CD49f, Trop2, c-Met and CK8. By analyzing EpCAM-depleted fractions from 25 metastatic breast cancer patients, we were able to identify 22, 27, 27 and 13 $EpCAM^{neg}$ CTCs applying enrichment for those 4 proteins. Together with targeting ADAM8, CD146, CD44 and HA, $EpCAM^{neg}$ CTCs could be detected in 69% of all analyzed samples. For the first time we can show that $EpCAM^{neg}$ cells captured by a CD44 antibody are of malignant nature.

In particular, CD49f (and CD146) was selected upon publications of Mostert *et al* in 2011 and 2012 [25, 26]. Herein CD49f, also designated as integrin α-6 adhesion molecule, had been implemented for a more sensitive CTC detection after a combined anti-EpCAM/CD146-enrichment. Besides its putative function as driver of metastasis [57], CD49f has been considered as stem cell marker in breast [58–60] and other solid tumors [61, 62]. In breast cancer, CD49f seems to be enriched in basal-like subtypes [63, 64], which is in concordance with our data from MDA-MB-231 showing the highest CD49f abundance, whereas its expression in luminal and HER2 subtypes was less pronounced. Trop2, a cell surface glycoprotein was implemented since it had been shown to be overexpressed in a majority of tumors [65] and to account for proliferation and invasion of tumor cells [66, 67]. Accessorily, when we started our study, Mikolajczyk *et al* had already published the use of a Trop2 (and also c-Met) antibody with regard to tumor cell enrichment via a micro-fluidic device [24]. Trop2 gained notice because it was expressed in all breast cancer cell lines examined, in contrast to EpCAM (= Trop1) expression. CD44, c-Met and CD47 were incorporated into our setup, inter alia, due to the findings of Baccelli and co-workers, who reported that CTCs possessing metastasis-initiating properties express CD44, c-Met and CD47 [28]. In a subsequent study they further showed that CD47 is a strong prognostic marker for luminal-type breast cancer patients, especially in co-expression with c-Met [68]. Our choice of CK8 was based on publications describing its cell surface expression in breast cancer cells, where it has been proposed to function as an important plasminogen binding-protein leading to increased cancer invasion [69–72]. Liu *et al* further

reported that membranous CK8 might be involved in cellular protection against chemotherapeutic treatment in multi-drug resistant MCF7 cells [73]. Within our study, we could also confirm CK8 surface expression via staining of unpermeabilized MCF7 cells and subsequent flow cytometry analysis (data not shown). While cell capturing on planar surfaces using a CK8 antibody failed, CTC enrichment with anti-CK8 beads was successful. Referring to ECM components, embedded into our enrichment approach, HA emerged as the most promising candidate, at least in terms of capturing MDA-MB-231 cells. It is widely accepted that cell-matrix-interactions are pivotal for intra- and extravasation of cells and thereof for promoting metastasis [74]. HA is one of the major components of the ECM and serves as a receptor for CD44 and RHAMM (receptor for HA-mediated motility) affecting diverse cellular processes (adhesion, migration, invasion) [75–77]. The assumption that mesenchymal EpCAM$^{low/neg}$ MDA-MB-231 cells express more CD44 and RHAMM compared to MCF7 (EpCAM^{pos}, epithelial) [78] underlines our observation that invasive MDA-MB-231 cells can be captured via HA.

Apart from identifying CTCs using our defined marker setup, supernumerary EpCAM^{neg} CK^{pos}/CD45^{pos} events could be detected in 28 out of 29 samples. The striking phenomenon of dual-positive (EpCAM/CK/EGFR and CD45 positive) cell detection has been observed by several groups before, while mostly being overlooked [79–82]. Consequently, the origin and occurrence of these cells, apparently combining epithelial and hematopoietic cell characteristics, still remains unclear. It has been debated that they result from fusions arising from interactions between tumor-infiltrating hematopoietic cells with epithelial cancer cells (`hemato-epithelial cells´) [83]. Other explanations might include false-positive CK^{pos} staining of leukocytes [84] or nonspecific antibody uptake owing to impaired membrane integrity. However, the fact that the number of dual-positive events is also remarkably high within the blood of healthy donors [84], and oddly, that a high proportion of those events could be assigned to patient samples with corresponding low EpCAM^{pos} CTC numbers, underscores the demand for further investigations with respect to their clinical significance.

Furthermore, the appearance of small cell clusters (2–3 cells) in EpCAM-depleted and bead-enriched fractions could be observed. This is in accordance with recent publications reporting about the detection of CTC clusters (2–50 cells), -mainly deriving from mesenchymal-like CTCs- in advanced cancers using different isolation platforms [21, 82]. Cho *et al* previously showed the presence of significant numbers of CTC aggregates in multiple epithelial cancers such as breast, prostate, lung, and pancreas [85]. Complementarily, Aceto *et al* state that these cell clusters might deduce from oligoclonal clumps of primary tumors representing a CTC subset with substantially increased metastatic capacities compared to their single counterparts in breast cancer. RNA sequencing further revealed that aggregate formation depends on the expression of plakoglobin, a member of the catenin protein family that contributes to desmosomes and adherens junctions [86, 87].

Being conscious of facing drawbacks in the light of unveiling new markers showing high specificity detection quality and quantity remain to be further studied, since those parameters, except for CK and EpCAM, are not well investigated yet. Further limitations include the unpredictability of blood sample compositions and epitope/antigen expression levels due to a high inter-patient variability. Using other markers for EpCAM-depleted patient sample analysis might have attained completely different results. This also accounts for our approach to identify EpCAM$^{low/neg}$ cells by CK positivity: we might have missed cells which have a fully developed mesenchymal phenotype and we are aware that EpCAM^{neg}/CK^{neg}/CD45^{neg} cells have to be investigated further for the expression of mesenchymal markers. Additionally, in order to elucidate distinct cell surface proteins that are associated with certain tumor characteristics, the patient cohort has to be further expanded regarding sample number and tumor subtypes.

Taken together, by targeting various cell surface proteins and ECM components, we were able to optimize the enrichment of heterogeneous EpCAM^{neg} expressing cancer cells as first proof of principle, whereas the highly flexible adaptability of our approach speaks in favor for the herein presented CTC enrichment strategy. Even though coverage of all CTC subpopulations will not be utterly achieved, further characterization of these identified EpCAM$^{pos/neg}$ and also dual-positive (CK^{pos}/CD45^{pos}) subsets on genomic/protein level is indispensable to determine an antibody cocktail which is evidently capturing cells of tumor origin. In the current status we cannot show clinical significance of our findings, but we were able to demonstrate the malignant nature of at least one EpCAM^{neg} subpopulation which harbors similar as well as different chromosomal aberrations in comparison to their EpCAM^{pos} counterpart. To prove clinical relevance of our approach a study identifying EpCAM$^{low/neg}$ CTC populations with verified aberrant genomic profiles should be designed.

Supporting Information

S1 Fig. Chip/array layout, slide chemistries and binding molecules of initial cell adhesion tests. Different NEXTERION slide chemistries (AL, A+, A*star*, E, NC-C, H) were spotted with 16 arrays consisting of spots with anti-EpCAM, anti-CK8, anti-Trop2, mouse (ms) isotype (blue, #1–4, triplicates) antibodies and fibronectin (FN), poly-L-lysin, vitronectin (VN), laminin (Lam), collagen I (Col), BSA (grey, #5–10; duplicates/triplicate for FN) (0.2 mg/ml each) and tested for binding of EpCAM^{pos} cells. Adhesion was visualized by Coomassie; 2x magnification.
(TIF)

S2 Fig. Total cell fluorescence of cell adhesion on multi-marker arrays. Total cell fluorescence (integral capture spot signals, in arbitrary units) of bound EpCAM^{pos} (MCF7, SKBR3, HCC1500, ZR-75-1, TMX2-28; left) and EpCAM$^{low/neg}$ (MDA-MB-231; right) cells (**see** Fig 3) was quantified by ImageJ/Fiji 1.46. Depicted are mean values of spot signals from three separate cell adhesion experiments.
(TIF)

S1 File. Recovery of SKBR3 cells by anti-EpCAM Adembeads.
(DOC)

S1 Table. The list of capture molecules spotted onto multi-marker arrays.
(XLS)

S2 Table. Overview of EpCAM-depleted samples, processed subfractions and used beads/markers per patient sample.
(XLS)

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Author Contributions

Conceived and designed the experiments: HS MP HN. Performed the experiments: HS BG FU. Analyzed the data: HS MP HN BB. Contributed reagents/materials/analysis tools: DN MP MFT TF NHS. Wrote the paper: HS NHS HN. Performed aCGH and analyzed data: BB.

References

1. Joosse SA, Gorges TM, Pantel K. Biology, detection, and clinical implications of circulating tumor cells. EMBO Molecular Medicine. 2014; 7(1):1–11. doi: 10.15252/emmm.201303698 PMID: 25398926
2. Zhang L, Riethdorf S, Wu G, Wang T, Yang K, Peng G, et al. Meta-Analysis of the Prognostic Value of Circulating Tumor Cells in Breast Cancer. Clinical Cancer Research. 2012; 18(20):5701–10. doi: 10.1158/1078-0432.CCR-12-1587 PMID: 22908097
3. Budd GT, Cristofanilli M, Ellis MJ, Stopeck A, Borden E, Miller MC, et al. Circulating Tumor Cells versus Imaging—Predicting Overall Survival in Metastatic Breast Cancer. Clinical Cancer Research. 2006; 12 (21):6403–9. doi: 10.1158/1078-0432.CCR-05-1769 PMID: 17085652
4. Bidard F-C, Peeters DJ, Fehm T, Nolé F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. The Lancet Oncology. 2014; 15(4):406–14. doi: 10.1016/S1470-2045(14)70069-5 PMID: 24636208
5. Pantel K, Alix-Panabières C. Circulating tumour cells in cancer patients: challenges and perspectives. Trends in Molecular Medicine. 2010; 16(9):398–406. doi: 10.1016/j.molmed.2010.07.001 PMID: 20667783
6. Butler TP, Gullino PM. Quantitation of Cell Shedding into Efferent Blood of Mammary Adenocarcinoma. Cancer research. 1975; 35(3):512–6. PMID: 1090362
7. Vanharanta S, Massagué J. Origins of Metastatic Traits. Cancer cell. 2013; 24(4):410–21. doi: 10.1016/j.ccr.2013.09.007 PMID: 24135279
8. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor Cells Circulate in the Peripheral Blood of All Major Carcinomas but not in Healthy Subjects or Patients With Nonmalignant Diseases. Clinical Cancer Research. 2004; 10(20):6897–904. doi: 10.1158/1078-0432.CCR-04-0378 PMID: 15501967
9. Coumans FAW, Ligthart ST, Uhr JW, Terstappen LWMM. Challenges in the Enumeration and Phenotyping of CTC. Clinical Cancer Research. 2012; 18(20):5711–8. doi: 10.1158/1078-0432.CCR-12-1585 PMID: 23014524
10. Barradas AMC, Terstappen LWMM. Towards the Biological Understanding of CTC: Capture Technologies, Definitions and Potential to Create Metastasis. Cancers. 2013; 5(4):1619–42. doi: 10.3390/cancers5041619 PMID: 24305653
11. Alix-Panabières C. EPISPOT Assay: Detection of Viable DTCs/CTCs in Solid Tumor Patients. In: Ignatiadis M, Sotiriou C, Pantel K, editors. Minimal Residual Disease and Circulating Tumor Cells in Breast Cancer. Recent Results in Cancer Research. 195: Springer Berlin Heidelberg; 2012. p. 69–76.
12. Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature. 2007; 450(7173):1235–9. PMID: 18097410
13. Lu J, Fan T, Zhao Q, Zeng W, Zaslavsky E, Chen JJ, et al. Isolation of circulating epithelial and tumor progenitor cells with an invasive phenotype from breast cancer patients. International Journal of Cancer. 2010; 126(3):669–83. doi: 10.1002/ijc.24814
14. Harb W, Fan A, Tran T, Danila DC, Keys D, Schwartz M, et al. Mutational Analysis of Circulating Tumor Cells Using a Novel Microfluidic Collection Device and qPCR Assay. Translational Oncology. 2013; 6 (5):528–38. PMID: 24151533
15. Choi H, Kim KB, Jeon CS, Hwang I, Lee S, Kim HK, et al. A label-free DC impedance-based microcytometer for circulating rare cancer cell counting. Lab on a Chip. 2013; 13(5):970–7. doi: 10.1039/C2LC41376K PMID: 23340965
16. Coumans FAW, van Dalum G, Beck M, Terstappen LWMM. Filter Characteristics Influencing Circulating Tumor Cell Enrichment from Whole Blood. PLoS ONE. 2013; 8(4):e61770. doi: 10.1371/journal.pone.0061770 PMID: 23626725
17. Kim YJ, Koo G-B, Lee J-Y, Moon H-S, Kim D-G, Lee D-G, et al. A microchip filter device incorporating slit arrays and 3-D flow for detection of circulating tumor cells using CAV1-EpCAM conjugated microbeads. Biomaterials. 2014; 35(26):7501–10. doi: 10.1016/j.biomaterials.2014.05.039 PMID: 24917030
18. Ozkumur E, Shah AM, Ciciliano JC, Emmink BL, Miyamoto DT, Brachtel E, et al. Inertial Focusing for Tumor Antigen–Dependent and–Independent Sorting of Rare Circulating Tumor Cells. Science Translational Medicine. 2013; 5(179):179ra47. doi: 10.1126/scitranslmed.3005616 PMID: 23552373
19. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002; 2(6):442–54. PMID: 12189386
20. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-Cadherin Promotes Metastasis via Multiple Downstream Transcriptional Pathways. Cancer research. 2008; 68(10):3645–54. doi: 10.1158/0008-5472.CAN-07-2938 PMID: 18483246

21. Tam WL, Weinberg RA. The epigenetics of epithelial-mesenchymal plasticity in cancer. Nat Med. 2013; 19(11):1438–49. doi: 10.1038/nm.3336 PMID: 24202396

22. Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science. 2013; 339(6119):580–4. doi: 10.1126/science.1228522 PMID: 23372014

23. Gorges T, Tinhofer I, Drosch M, Rose L, Zollner T, Krahn T, et al. Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. BMC Cancer. 2012; 12(1):178. doi: 10.1186/1471-2407-12-178

24. Mikolajczyk SD, Millar LS, Tsinberg P, Coutts SM, Zomorrodi M, Pham T, et al. Detection of EpCAM-Negative and Cytokeratin-Negative Circulating Tumor Cells in Peripheral Blood. Journal of Oncology. 2011; 2011:252361. doi: 10.1155/2011/252361 PMID: 21577258

25. Mostert B, Kraan J, Bolt-de Vries J, van der Spoel P, Sieuwerts A, Schutte M, et al. Detection of circulating tumor cells in breast cancer may improve through enrichment with anti-CD146. Breast Cancer Research and Treatment. 2011; 127(1):33–41. doi: 10.1007/s10549-010-0879-y PMID: 20379845

26. Mostert B, Kraan J, Sieuwerts AM, van der Spoel P, Bolt-de Vries J, Prager-van der Smissen WJC, et al. CD49f-based selection of circulating tumor cells (CTCs) improves detection across breast cancer subtypes. Cancer Letters. 2012; 319(1):49–55. doi: 10.1016/j.canlet.2011.12.031 PMID: 22202642

27. Obermajer N, Doljak B, Kos J. Cytokeratin 8 ectoplasmic domain binds urokinase-type plasminogen activator to breast tumor cells and modulates their adhesion, growth and invasiveness. Molecular Cancer. 2009; 8(1):88.

28. Baccelli I, Schneeweiss A, Riethdorf S, Stenzinger A, Schillert A, Vogel V, et al. Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. Nature biotechnology. 2013; 31(6):539–44. doi: 10.1038/nbt.2576 PMID: 23609047

29. Romagnoli M, Mineva ND, Polmear M, Conrad C, Srinivasan S, Loussouarn D, et al. ADAM8 expression in invasive breast cancer promotes tumor dissemination and metastasis. EMBO Molecular Medicine. 2013; 6(2):278–94. doi: 10.1002/emmm.201303373 PMID: 24375628

30. Chen D, Bhat-Nakshatri P, Goswami C, Badve S, Nakshatri H. ANTXR1, a stem cell-enriched functional biomarker, connects collagen signaling to cancer stem-like cells and metastasis in breast cancer. Cancer research. 2013; 73(18):5821–33. doi: 10.1158/0008-5472.CAN-13-1080 PMID: 23832666

31. Gozgit JM, Pentecost BT, Marconi SA, Otis CN, Wu C, Arcaro KF. Use of an Aggressive MCF-7 Cell Line Variant, TMX2-28, to Study Cell Invasion in Breast Cancer. Molecular Cancer Research. 2006; 4 (12):905–13. doi: 10.1158/1541-7786.MCR-06-0147 PMID: 17189381

32. Schneck H, Blassl C, Meier-Stiegen F, Neves RP, Janni W, Fehm T, et al. Analysing the mutational status of PIK3CA in circulating tumor cells from metastatic breast cancer patients. Molecular oncology. 2013; 7(5):976–86. doi: 10.1016/j.molonc.2013.07.007 PMID: 23895914

33. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Meth. 2012; 9(7):676–82.

34. Möhlendick B, Bartenhagen C, Behrens B, Honisch E, Raba K, Knoefel WT, et al. A Robust Method to Analyze Copy Number Alterations of Less than 100 kb in Single Cells Using Oligonucleotide Array CGH. PLoS One. 2013; 8(6):e67031. doi: 10.1371/journal.pone.0067031 PMID: 23825608

35. Neves RPL, Raba K, Schmidt O, Honisch E, Meier-Stiegen F, Behrens B, et al. Genomic High-Resolution Profiling of Single CKpos/CD45neg Flow-Sorting Purified Circulating Tumor Cells from Patients with Metastatic Breast Cancer. Clinical Chemistry. 2014; 60(10):1290–7. doi: 10.1373/clinchem.2014.222331 PMID: 25267515

36. Möhlendick B, Stoecklein N. Analysis of Copy-Number Alterations in Single Cells Using Microarray-Based Comparative Genomic Hybridization (aCGH). Curr Protoc Cell Biol. 2014; 1(65):22.19.1–22.19.23. doi: 10.1002/0471143030.cb2219s65

37. Gancberg D, Di Leo A, Cardoso F, Rouas G, Pedrocchi M, Paesmans M, et al. Comparison of HER-2 status between primary breast cancer and corresponding distant metastatic sites. Annals of Oncology. 2002; 13(7):1036–43. doi: 10.1093/annonc/mdf252 PMID: 12176781

38. Meng S, Tripathy D, Frenkel EP, Shete S, Naftalis EZ, Huth JF, et al. Circulating Tumor Cells in Patients with Breast Cancer Dormancy. Clinical Cancer Research. 2004; 10(24):8152–62. doi: 10.1158/1078-0432.CCR-04-1110 PMID: 15623589

39. Mueller V, Pantel K. HER2 as marker for the detection of circulating tumor cells. Breast Cancer Research and Treatment. 2009; 117(3):535–7. doi: 10.1007/s10549-008-0276-y PMID: 19115105

40. Powell AA, Talasaz AH, Zhang H, Coram MA, Reddy A, Deng G, et al. Single cell profiling of Circulating tumor cells: Transcriptional heterogeneity and diversity from breast cancer cell lines. PLoS ONE. 2012; 7(5):e33788. doi: 10.1371/journal.pone.0033788 PMID: 22586443

41. Qu Q, Zong Y, Fei X-c, Chen X-s, Xu C, Lou G-y, et al. The importance of biopsy in clinically diagnosed metastatic lesions in patients with breast cancer. World Journal of Surgical Oncology. 2014; 12:93. doi: 10.1186/1477-7819-12-93 PMID: 24721777

42. Gasch C, Bauernhofer T, Pichler M, Langer-Freitag S, Reeh M, Seifert AM, et al. Heterogeneity of Epidermal Growth Factor Receptor Status and Mutations of KRAS/PIK3CA in Circulating Tumor Cells of Patients with Colorectal Cancer. Clinical Chemistry. 2013; 59(1):252–60. doi: 10.1373/clinchem.2012.188557 PMID: 23136247

43. Balzar M, Winter MJ, de Boer CJ, Litvinov SV. The biology of the 17–1A antigen (Ep-CAM). Journal of Molecular Medicine. 1999; 77(10):699–712. doi: 10.1007/s001099900038 PMID: 10606205

44. Went PTH, Lugli A, Meier S, Bundi M, Mirlacher M, Sauter G, et al. Frequent EpCam protein expression in human carcinomas. Human Pathology. 2004; 35(1):122–8. doi: 10.1016/j.humpath.2003.08.026 PMID: 14745734

45. Massoner P, Thomm T, Mack B, Untergasser G, Martowicz A, Bobowski K, et al. EpCAM is overexpressed in local and metastatic prostate cancer, suppressed by chemotherapy and modulated by MET-associated miRNA-200c/205. Br J Cancer. 2014; 111(5):955–64. doi: 10.1038/bjc.2014.366 PMID: 24992580

46. Tai KY, Shiah SG, Shieh YS, Kao YR, Chi CY, Huang E, et al. DNA methylation and histone modification regulate silencing of epithelial cell adhesion molecule for tumor invasion and progression. Oncogene. 2007; 26(27):3989–97. PMID: 17213811

47. Punnoose EA, Atwal SK, Spoerke JM, Savage H, Pandita A, Yeh R-F, et al. Molecular Biomarker Analyses Using Circulating Tumor Cells. PLoS ONE. 2010; 5(9):e12517. doi: 10.1371/journal.pone.0012517 PMID: 20838621

48. Thurm H, Ebel S, Kentenich C, Hemsen A, Riethdorf S, Coith C, et al. Rare Expression of Epithelial Cell Adhesion Molecule on Residual Micrometastatic Breast Cancer Cells after Adjuvant Chemotherapy. Clinical Cancer Research. 2003; 9(7):2598–604. PMID: 12855636

49. Gires O, Stoecklein NH. Dynamic EpCAM expression on circulating and disseminating tumor cells: causes and consequences. Cellular and Molecular Life Sciences. 2014; 71(22):4393–402. doi: 10.1007/s00018-014-1693-1 PMID: 25103341

50. Patriarca C, Macchi RM, Marschner AK, Mellstedt H. Epithelial cell adhesion molecule expression (CD326) in cancer: A short review. Cancer Treatment Reviews. 2012; 38(1):68–75. doi: 10.1016/j.ctrv.2011.04.002 PMID: 21576002

51. Songun I, Litvinov SV, van de Velde CJH, Pals ST, Hermans J, van Krieken JHJM. Loss of Ep-CAM (CO17-1A) expression predicts survival in patients with gastric cancer. Br J Cancer. 2005; 92(9):1767–72. PMID: 15870832

52. van der Gun BTF, Melchers LJ, Ruiters MHJ, de Leij LFMH, McLaughlin PMJ, Rots MG. EpCAM in carcinogenesis: the good, the bad or the ugly. Carcinogenesis. 2010; 31(11):1913–21. doi: 10.1093/carcin/bgq187 PMID: 20837599

53. Rao CG, Chianese D, Doyle GV, Miller MC, Russell T, Sanders RA, et al. Expression of epithelial cell adhesion molecule in carcinoma cells present in blood and primary and metastatic tumors. International Journal of Oncology. 2005; 27(1):49–57. PMID: 15942643

54. Vannier C, Mock K, Brabletz T, Driever W. Zeb1 Regulates E-cadherin and Epcam (Epithelial Cell Adhesion Molecule) Expression to Control Cell Behavior in Early Zebrafish Development. Journal of Biological Chemistry. 2013; 288(26):18643–59. doi: 10.1074/jbc.M113.467787 PMID: 23667256

55. Gosens MJEM, van Kempen LCL, van de Velde CJH, van Krieken JHJM, Nagtegaal ID. Loss of membranous Ep-CAM in budding colorectal carcinoma cells. Mod Pathol. 2007; 20(2):221–32. PMID: 17361206

56. Driemel C, Kremling H, Schumacher S, Will D, Wolters J, Lindenlauf N, et al. Context-dependent adaption of EpCAM expression in early systemic esophageal cancer. Oncogene. 2014; 33(41):4904–15. doi: 10.1038/onc.2013.441 PMID: 24141784

57. Wang Y, Shenouda S, Baranwal S, Rathinam R, Jain P, Bao L, et al. Integrin subunits alpha5 and alpha6 regulate cell cycle by modulating the chk1 and Rb/E2F pathways to affect breast cancer metastasis. Molecular Cancer. 2011; 10(1):84.

58. Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM, Goodell MA. Sca-1pos Cells in the Mouse Mammary Gland Represent an Enriched Progenitor Cell Population. Developmental Biology. 2002; 245(1):42–56. doi: 10.1006/dbio.2002.0625 PMID: 11969254

59. Cariati M, Naderi A, Brown JP, Smalley MJ, Pinder SE, Caldas C, et al. Alpha-6 integrin is necessary for the tumourigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line. International Journal of Cancer. 2008; 122(2):298–304. doi: 10.1002/ijc.23103

60. Honeth G, Bendahl P-O, Ringnér M, Saal LH, Gruvberger-Saal SK, Lövgren K, et al. The CD44 +/CD24- phenotype is enriched in basal-like breast tumors. Breast Cancer Research: BCR. 2008; 10 (3):R53. doi: 10.1186/bcr2108 PMID: 18559090

61. Lawson DA, Xin L, Lukacs RU, Cheng D, Witte ON. Isolation and functional characterization of murine prostate stem cells. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104(1):181–6. doi: 10.1073/pnas.0609684104 PMID: 17185413

62. Grossman HB, Lee C, Bromberg J, Liebert M. Expression of the alpha6beta4 integrin provides prognostic information in bladder cancer. Oncology Reports. 2000; 7(1):13–9. PMID: 10601583

63. Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, et al. Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat Med. 2009; 15(8):907–13. doi: 10.1038/nm.2000 PMID: 19648928

64. Vieira AF, Ricardo S, Ablett MP, Dionísio MR, Mendes N, Albergaria A, et al. P-Cadherin Is Coexpressed with CD44 and CD49f and Mediates Stem Cell Properties in Basal-like Breast Cancer. STEM CELLS. 2012; 30(5):854–64. doi: 10.1002/stem.1075 PMID: 22389315

65. Fornaro M, Dell'Arciprete R, Stella M, Bucci C, Nutini M, Capri MG, et al. Cloning of the gene encoding Trop-2, a cell-surface glycoprotein expressed by human carcinomas. Int J Cancer. 1995; 65(5):610–8.

66. Guerra E, Trerotola M, Aloisi AL, Tripaldi R, Vacca G, La Sorda R, et al. The Trop-2 signalling network in cancer growth. Oncogene. 2013; 32(12):1594–600. doi: 10.1038/onc.2012.151 PMID: 22562244

67. Trerotola M, Jernigan DL, Liu Q, Siddiqui J, Fatatis A, Languino LR. Trop-2 promotes prostate cancer metastasis by modulating β(1) integrin functions. Cancer research. 2013; 73(10):3155–67. doi: 10.1158/0008-5472.CAN-12-3266 PMID: 23536555

68. Baccelli I, Stenzinger A, Vogel V, Maria Pfitzner B, Klein C, Wallwiener M, et al. Co-expression of MET and CD47 is a novel prognosticator for survival of luminal-type breast cancer patients. Oncotarget. 2014; 5(18):8147–60. PMID: 25230070

69. Hembrough TA, Vasudevan J, Allietta MM, Glass WF, Gonias SL. A cytokeratin 8–like protein with plasminogen-binding activity is present on the external surfaces of hepatocytes, HepG2 cells and breast carcinoma cell lines. J Cell Sci. 1995; 108:1071–82. PMID: 7542667

70. Hembrough TA, Li L, Gonias S. Cell-surface cytokeratin 8 is the major plasminogen receptor on breast cancer cells and is required for the accelerated activation of cell-associated plasminogen by tissue-type plasminogen activator. J Biol Chem. 1996; 271:25684–91. PMID: 8810346

71. Godfroid E, Geuskens M, Dupressoir T, Parent I, Szpirer C. Cytokeratins are exposed on the outer surface of established human mammary carcinoma cells. Journal of Cell Science. 1991; 99(3):595–607.

72. Gonias S, Hembrough T, Sankovic M. Cytokeratin 8 functions as a major plasminogen receptor in select epithelial and carcinoma cells. Front Biosci. 2001; 6:D1403–11. PMID: 11689350

73. Liu F, Chen Z, Wang J, Shao X, Cui Z, Yang C, et al. Overexpression of Cell Surface Cytokeratin 8 in Multidrug-Resistant MCF-7/MX Cells Enhances Cell Adhesion to the Extracellular Matrix. Neoplasia (New York, NY). 2008; 10(11):1275–84.

74. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell. 2011; 144(5):646–74. doi: 10.1016/j.cell.2011.02.013 PMID: 21376230

75. Brown TJ, Thomas NK. Role of Hyaluronan Metabolism in the Initiation, Invasion, and Metastasis of Breast Cancer. In: Stern RBT, editor. Hyaluronan in Cancer Biology. San Diego: Academic Press; 2009.

76. Peach RJ, Hollenbaugh D, Stamenkovic I, Aruffo A. Identification of hyaluronic acid binding sites in the extracellular domain of CD44. The Journal of Cell Biology. 1993; 122(1):257–64. doi: 10.1083/jcb.122.1.257 PMID: 8314845

77. Wang C, Thor AD, Moore DH, Zhao Y, Kerschmann R, Stern R, et al. The overexpression of RHAMM, a hyaluronan-binding protein that regulates ras signaling, correlates with overexpression of mitogen-activated protein kinase and is a significant parameter in breast cancer progression. Clinical Cancer Research. 1998; 4(3):567–76. PMID: 9533523

78. Hamilton SR, Fard SF, Paiwand FF, Tolg C, Veiseh M, Wang C, et al. The hyaluronan receptors CD44 and RHAMM (CD168) form Complexes with ERK1/2, which sustain high basal motility in breast cancer cells. The Journal of biological chemistry. 2007; 282(22):16667–80. doi: 10.1074/jbc.M702078200 PMID: 17392272

79. Yu M, Stott S, Toner M, Maheswaran S, Haber DA. Circulating tumor cells: approaches to isolation and characterization. The Journal of Cell Biology. 2011; 192(3):373–82. doi: 10.1083/jcb.201010021 PMID: 21300848

80. Nel I, Jehn U, Gauler T, Hoffmann A-C. Individual profiling of circulating tumor cell composition in patients with non-small cell lung cancer receiving platinum based treatment. Translational Lung Cancer Research. 2014; 3(2):100–6. doi: 10.3978/j.issn.2218-6751.2014.03.05 PMID: 25806288

81. Khoo BL, Warkiani ME, Tan DS-W, Bhagat AAS, Irwin D, Lau DP, et al. Clinical Validation of an Ultra High-Throughput Spiral Microfluidics for the Detection and Enrichment of Viable Circulating Tumor Cells. PLoS ONE. 2014; 9(7):e99409. doi: 10.1371/journal.pone.0099409 PMID: 24999991

82. Stott SL, Hsu C-H, Tsukrov DI, Yu M, Miyamoto DT, Waltman BA, et al. Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107(43):18392–7. doi: 10.1073/pnas.1012539107 PMID: 20930119

83. Ramakrishnan M, Mathur SR, Mukhopadhyay A. Fusion-Derived Epithelial Cancer Cells Express Hematopoietic Markers and Contribute to Stem Cell and Migratory Phenotype in Ovarian Carcinoma. Cancer research. 2013; 73(17):5360–70. doi: 10.1158/0008-5472.CAN-13-0896 PMID: 23856249

84. Fischer JC, Niederacher D, Topp SA, Honisch E, Schumacher S, Schmitz N, et al. Diagnostic leukapheresis enables reliable detection of circulating tumor cells of nonmetastatic cancer patients. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(41):16580–5. doi: 10.1073/pnas.1313594110 PMID: 24065821

85. Cho EH, Wendel M, Luttgen M, Yoshioka C, Marrinucci D, Lazar D, et al. Characterization of circulating tumor cell aggregates identified in patients with epithelial tumors. Physical Biology. 2012; 9(1):16001. doi: 10.1088/1478-3975/9/1/016001

86. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, et al. Circulating Tumor Cell Clusters Are Oligoclonal Precursors of Breast Cancer Metastasis. Cell. 2014; 158(5):1110–22. doi: 10.1016/j.cell.2014.07.013 PMID: 25171411

87. Harris TJC, Tepass U. Adherens junctions: from molecules to morphogenesis. Nature Reviews Molecular Cell Biology. 2010; 11:502–14. doi: 10.1038/nrm2927 PMID: 20571587

3.2 Review

3.2.1 Prognostic Relevance of Circulating Tumor Cells in Molecular Subtypes of Breast Cancer

Prognostische Relevanz zirkulierender Tumorzellen in molekularen Subtypen des Mammakarzinoms

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Prognostic Relevance of Circulating Tumor Cells in Molecular Subtypes of Breast Cancer

Prognostische Relevanz zirkulierender Tumorzellen in molekularen Subtypen des Mammakarzinoms

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Schlüsselwörter
Mammakarzinom
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molekularer Subtyp
Prognose

Abstract

Circulating tumor cells (CTCs) can be detected in the peripheral blood of breast cancer patients with early and metastatic disease. Recent data suggest that immune pathologic characteristics between the primary tumor, metastatic colonies and CTCs are discordant and that CTCs possess an independent phenotype that is associated with prognosis and treatment efficacy. Large scale gene expression analysis has provided the possibility to stratify breast cancer according to the gene expression fingerprint of primary tumor tissue into five intrinsic molecular subtypes which can be associated with different clinical outcome. As a consequence of the different prognostic power of primary tumors' characteristics and CTCs several groups have started to investigate if CTCs might be disseminated differentially within these breast cancer subtypes. They determined the CTC number in immunohistochemical sub- types to validate if CTCs may provide differential and more specific prognostic information within each subtype. This review provides an overview of the outcome of some recently published data gathered from early and metastatic breast cancer.

Zusammenfassung

Zirkulierende Tumorzellen (CTC) können im Blut von Mammakarzinom-Patientinnen in der adjuvanten sowie metastatischen Situation detektiert werden. Bezüglich des Immunphänotyps können sich CTC sowohl von den Metastasen als auch vom Primärtumor unterscheiden. Moderne Genexpressionsanalyse lässt Mammakarzinome in 5 molekulare Subtypen mit unterschiedlichem biologischem und klinischem Verhalten unterteilen. Aufgrund der unterschiedlichen prognostischen Bedeutung zirkulierender Tumorzellen und der prädiktiven Faktoren beim Mammakarzinom wurde das Phänomen der Tumorzelldissemination innerhalb der verschiedenen Mammakarzinom-Subtypen bereits von vielen Gruppen analysiert. Die CTC-Anzahl wurde bei Tumoren mit unterschiedlicher Immunhistochemie bestimmt und auf ihre prognostische Aussagekraft in Abhängigkeit von der Biologie des Primärtumors hin untersucht. Dieser Review bietet einen Überblick über aktuelle Daten im adjuvanten und metastasierten Setting.

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Abbreviations

BCSS	breast cancer-specific survival CK cytokeratin
CTC	circulating tumor cell
DFS	disease-free survival
EMT	epithelial-mesenchymal transition ER estrogen receptor
FISH	fluorescence in situ hybridization HR hormone receptor
IHC	immunohistochemistry
MBC	metastatic breast cancer
MFS	metastasis-free survival
OS	overall survival
PFS	progression-free survival
TN	triple-negative

Introduction

Heterogeneity of breast cancer

Breast cancer should not be considered as a single heterogeneous disease but as a conglomerate of heterogeneous diseases consisting of a plethora of different molecular histopathologic subtypes, clinical outcomes and responses to therapies. Currently, for patients with operable breast cancer the status of routine pathologic parameters determined in the primary tumor such as tumor size, lymph node status,

endocrine receptor status, and the HER2 status are used to estimate risk and to give recommendations for adjuvant therapy. Improvements in determining these pathologic parameters as well as adjusted and targeted therapies have resulted in better prediction and prognosis, however, a significant proportion of cancer patients may be overtreated.

Approximately 20 % of breast cancer patients who initially present with a localized disease will progress to metastatic breast cancer (MBC) and die of metastases [1]. Clinical management of patients with MBC is much less comprehensively structured. Its treatment is frequently based on expression profiles of the primary tumor. However, basing treatment decisions solely on the morphological features of the primary tumor potentially ignores many biological features of the metastases that may affect outcome [2, 3].

It is unclear whether different subclones of a heterogeneous primary tumor will metastasize to different organs, or if the expression of biomarkers within the metastasis will change due to adjuvant treatment or targeted therapy [4]. Many authors have noted a discrepancy between primary tumor and metastatic sites with regard to HER2 and hormone receptor (HR) status [5–8]. Routine biopsies of metastatic lesions are strongly recommended but sometimes hard to provide or somewhat dangerous for the patient. Nevertheless serial reevaluation of metastatic disease with regard to HER2 and hormone receptor status would be interesting from a scientific standpoint and could help to optimize treatment decisions.

These unsatisfying situations, both in the primary and in the metastatic disease, warrant to tune our available prognostic and predictive tests such as the information on intrinsic breast cancer subtypes and the presence of circulating tumor cells (CTCs) to potentially prevent patients from receiving unnecessary and/or ineffective treatment but experiencing the treatment-related side effects.

In the last decade progress in large scale molecular characterization of cancer tissues has extended our knowledge on heterogeneity of e.g. breast cancer to new levels [9]. In their seminal work Perou and coworkers provided the basis to associate the phenotypic diversity of primary breast tumors with corresponding gene expression profiles and enabled to classify breast cancers into four – later on five – different molecular subtypes of breast cancer: estrogen receptor (ER)positive (ER+)/luminal-A or -B, basal-like, HER2+, and normal breast [10, 11]. The existence of these five molecular subtypes has later been confirmed in independent datasets [12] and besides that they have been associated with significant differences in distant spread patterns, independent of conventional clinical–pathologic variables [13].

These classified molecular subtypes which were originally based on the differential expression of a set of genes have recently been transferred into a surrogate clinico-pathologic characterization based on ER/PR expression, HER2 status, proliferation or histologic grade using immune histochemical (IHC) techniques [14, 15]. This prompted experts taking part in the 2011 St. Gallen Consensus Conference to introduce this surrogate definition of intrinsic breast cancer subtypes (luminal A-like, luminal B-like [HER2-], luminal B-like [HER2+], HER2, and basal-like) into clinical use to better define the categories of breast cancers to be treated [16].

The concept of circulating tumor cells

The theory on hematogenous spread of cancer was developed by several researchers in 19th century [17]. It is now widely accepted that solid tumors are able to shed single tumor cells into the blood circulation where they are dispersed throughout the body [18–20]. Those CTCs may suffer different fates: they go into apoptosis, fall into a dormant state (dormancy) or they survive and finally transmigrate into secondary organ sites to persist and to eventually outgrow to metastases [21, 22]. This transient nature of a single CTC in the peripheral blood makes it an attractive candidate to take a screenshot of the current expression of therapeutically relevant markers in the potentially harmful cell population in a minimally-invasive liquid biopsy format. Highly sensitive methods based on expression of surface proteins or their physical characteristics have been developed in recent years to detect single CTC for diagnostic purposes. One such method is utilized by the CellSearch System (Veridex, LLC, Warren, NJ, USA), a device approved by the US Food and Drug Administration (FDA) in 2004 for the detection of CTCs in the peripheral blood of patients with MBC. Another approach is to perform RT-PCR measurement of cytokeratin (CK) mRNA expression levels (CK19, CK20) which are used as surrogate for the presence of CTCs in the peripheral blood and which have been shown to correlate with disease outcome and the results from CellSearch [23, 24]. The great hope regarding the CTCs' clinical relevance is that by characterizing their individual phenotype and/or genotype clinicians will be able to target the Achilles heel of these CTCs which are finally responsible for metastasis in order to improve prognosis and prediction.

Prognostic Relevance of CTCs

Metastatic breast cancer

In advanced breast cancer the CTCs' prognostic value has clearly been demonstrated – particularly their detection before treatment as an independent predictor of progression-free survival (PFS) and overall survival (OS) [25–27]. Moreover, a substantial decrease in the CTC count is an early marker of individual response to treatment and thus CTC screening provides an easy- to-perform alternative method to monitor success of a given therapy [28].

Since CTC levels appear to reflect response to treatment early in the course of a new regimen, they might potentially guide therapy decisions in metastatic breast cancer. This issue has been addressed by two currently ongoing clinical trials: SWOG 0500 from the Southwestern Oncology Group and CirCe01 at the Institut Curie, France [29, 30]. In CirCe01, patients in the advanced metastatic setting (3rd line and higher) with insufficient CTC decrease after start of new therapy line will change to another regimen, which will be, again, evaluated by early CTC changes. In the SWOG 0500 phase III trial, patients with persistently elevated CTC counts after one cycle of first-line chemotherapy were randomized between continuation of treatment until clinical progression or to early switch to another regimen [30]. Both studies were designed to clarify whether CTC changes may detect chemoresistance earlier than classical radiological methods and save patients with metastatic disease from the adverse effects of an inefficient chemotherapy regimen.

The first results of the SWOG 0500 trial, presented at the San Antonio Breast Cancer Symposium 2013 and now published in the Journal of Clinical Oncology, show that switching to another

chemotherapy regimen does not improve survival in patients with elevated CTC counts after one cycle of initial chemotherapy [30]. The SWOG 0500 trial confirmed also that patients with low baseline CTC levels perform best, reaching a median overall survival of 35 months, followed by patients whose CTC levels decrease during treatment (23 months) and patients with persistently high CTC levels (13 months). Which patients need first-line chemotherapy or – in case of HR-positive disease – only endocrine treatment will be addressed by the French STIC CTC Metabreast trial (NCT01710605) and the USA/Canada-based COMETI-P2 trial (NCT01701050). In the STIC CTC trial, patients were randomized between clinician choice and CTC-driven choice; patients in the "CTC arm" will be stratified to chemo- or endocrine treatment based on their CTC counts (high levels: chemotherapy, low levels: endocrine therapy). Treatment in the standard arm will be based on clinicians' decision. The COMETI-P2 study is a phase II trial evaluating the feasibility of the CTC-Endocrine Therapy Index. This index is based on the expression of four markers (estrogen receptor, Bcl2, HER2, and Ki67) assessed on isolated CTCs by immunocytofluorescence (CellSearch®) and was designed to predict clinical response to endocrine treatment in metastatic setting [31].

Early breast cancer

Presence of CTCs has been reported in 10–60 % of patients with stage I–III breast cancer by various types of detection assays [32, 33], e.g. CK19 mRNA amplification [23] or the CellSearch (Veridex, Raritan, NJ) method [34]. Recently, Rack et al. published the results from the German SUCCESS trial; blood samples from 2026 early average-to-high risk breast cancer patients before chemo- therapy and 1492 patients after chemotherapy were analyzed [33]. The presence of CTCs was strongly associated with shorter disease-free survival (DFS) and OS. Further, patients with at least 5 CTCs/30 ml blood were at highest risk for disease recurrence. Patients with CTC persistence after chemotherapy had significantly worse DFS and OS as well. These new data from a large clinical trial support previous findings from the REMAGUS 02 trial which reported that the presence of one or more CTCs before the start of systemic chemotherapy is an independent predictor of both metastasis-free survival (MFS) and OS in patients with stage II and III breast cancer [35]. In congruence with these findings are data from smaller studies [36, 37].

Prognostic Relevance of CTCs According to Breast Cancer Subtype

Recent data suggest that the phenotypes and several clinico-pathologic characteristics are discordant among the primary tumor, metastatic cells, and isolated tumor cells [5–7, 38, 39]. This indicates that it may be the presence of CTCs or even the phenotype/genotype of an individual CTC which is associated with breast cancer prognosis and treatment response in the first place [40]. Therefore, detection, (molecular) characterization, and the clinical role of CTCs in different subtypes of breast cancer are currently investigated in several research projects.

Metastatic breast cancer

In the first study addressing this question, Giordano et al. retrospectively analyzed 517 MBC patients for the presence of CTCs [41] (Table 1). Subtypes of primary tumors were determined by immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH) and CTCs were enumerated by CellSearch before start of a new systemic treatment. The authors classified patients with at least one positive hormone receptor (HR; ER or PR ≥ 1%) as HR+. 206 (40%) patients had ≥ 5 CTCs at baseline blood draw, 311 (60%) had < 5 CTCs. Regarding overall distribution of CTCs in different subtypes, a larger proportion of HR+/HER2-negative patients had ≥ 5 CTCs than did patients with other subtypes of tumor – a finding which does not overlap with previously published results from similar but smaller population of MBC patients [42–44]. The prognostic power of the CTC count appeared to be most valuable in HR+ and TN breast cancers and least valuable in HER2-positive cancers pretreated with targeted therapy. HER2-positive MBC patients with ≥ 5 CTCs had a PFS and an OS similar to patients with < 5 CTCs which indicates that CTCs are strongly predictive for survival in all HER2-negative MBC subtypes who had not been treated with targeted therapy.

A second study using CellSearch to determine the CTC count in MBC patients was published by Munzone et al. [45]. This group retrospectively classified 203 patients into intrinsic subgroups defined by IHC of 5 biomarkers (ER, PR, HER2, Ki-67, and grade), according to the recent St. Gallen guidelines, and determined their CTC status. All patients had CTCs enumerated before starting a new treatment as standard of care. Most patients (74 %) were pretreated for metastatic disease and had more than one metastatic site (66%). In total, 92 patients had ≥ 5 CTCs/7.5 ml blood and again at multivariable analysis, CTC count was a statistically significant predictor for PFS and OS confirming the CTCs' prognostic power in MBC. When stratifying the tumors into the intrinsic subgroups the results are confirming CTCs as strong predictor of survival in different MBC subtypes:

Table 1. CTCs and molecular subtypes in metastatic breast cancer.

Author	n (≥ 5 CTC)	Method	Frequency bias	All subtypes	Lum A	Lum B-like		HER2+	TN
						HER2−	HER2+		
Giordano (2012)	517 (206)	CS	HR+/HER 2−	PFS, OS	PFS, OS		n. s. (OS p = 0.084)	n. s.	OS
Munzone (2012)	203 (92)	CS	HR+/HER 2−	PFS, OS	PFS, OS	OS	OS	OS	n. s. (OS p = 0.06)
Wallwiener (2013)	468 (205)	CS	ER+	PFS, OS		PFS, OS	OS		PFS, OS

CS: CellSearch™; HR: hormone receptor; PFS: progression free survival; OS: overall survival; n. s.: not significant

CTCs were mostly found in patients with luminal-A/luminal-B/HER2-negative subtypes. The CTC count was a significant prognostic factor for OS in all subtypes, except for triple negative patients (borderline significance). In the HR+/HER2-negative patients CTC count was a significant prognostic factor for PFS.

The most recent data come from a large prospective multicenter study including nine German breast cancer centers. Therein Wallwiener et al. investigated blood samples from 486 MBC patients [46]. The study design was very similar to the one followed by Giordano et al. [41] with the molecular subtypes being determined by immunohistochemical staining of the primary tumor. CTC enumeration was carried out before initiation of a new therapy using the CellSearch system obtaining 205 CTC-positive patients. Like Giordano et al., the authors classified patients which were positive for estrogen and/or progesterone as HR+ and classified the patients into three subgroups

1. HR+/HER2−,
2. HER2+, or
3. HR−/HER2− (triple negative).

As already observed by Giordano et al. [41], CTC-positivity was significantly more frequent in ER+ patients and the CTC status did not differ significantly among subtypes of MBC. Additionally, Wallwiener et al. observed a higher rate of CTC-positivity in patients with both bone and visceral metastases compared to those with either bone or visceral metastases. When stratified by the molecular subtype, baseline CTC count was predictive for OS in all subgroups. Regarding PFS, the CTC status was a prognostic factor in HR+/HER2− and HR−/HER2− patients. Wallwiener et al. separated the patients with HER2+ primary tumors according to their pretreatment with trastuzumab and found out that the CTC status of untreated HER2+ patients was prognostic for OS [46]. No impact on OS was observed in HER2+ patients with previous trastuzumab treatment. No prognostic effect of CTC for PFS was observed in both groups.

Regarding the prognostic significance of CTC in MBC subtypes there seem to be two common themes. In general CTCs are more often found in luminal A (HR+/HER2−) and luminal B/HER2− tumors compared with HER2+ and basal-like tumors. This may be a consequence of the clinical practice that HER2+ MBC patients are treated with HER2-targeted therapy (trastuzumab, lapatinib) [45]. Alternatively, it is known that cells of basal-like molecular breast cancer subtype may shift to low EpCAM expression and increased expression of mesenchymal markers such as vimentin, epidermal growth factor receptor (EGFR) and epithelial-mesenchymal-transition (EMT) compared to breast cancers with a luminal subtype [47, 48]. Since CellSearch™ is a purely EpCAM-based capture system it may identify less basal-like cells. This may bias their frequency in a way that the number of basal-like CTCs might be underestimated [49, 50]. Secondly, in most studies, CTC detection did not predict clinical outcome in women with HER2+ MBC [41]. Fittingly, Munzone et al. observed that patients with 0 CTCs/7.5 mL blood at baseline and all subtypes, except for HER2+, seemed to perform better than CTC-positive patients [45]. Further studies demonstrated a marked decrease in CTC count at follow-up if MBC patients had received biological therapies such as trastuzumab or bevacizumab [42, 44]. In the studies published by Munzone et al. [45] and Giordano et al. [41] most of the HER2+ breast cancer patients have received anti-HER2 treatment which may have eliminated CTCs with HER2 amplification or overexpression and may thereby have reduced the prognostic value of CTC enumeration in this subtype. In support, Georgoulias et al. [51] showed that trastuzumab eliminates chemotherapy-resistant CTCs and reduces the risk of dis- ease recurrence in early breast cancer patients. In the study from Wallwiener et al. the number of patients treated with trastuzumab was quite low (6.5 %), whereas CTC detection had no impact on survival if patients had already received trastuzumab treatment [46]. CTC detection was a predictor of OS in the subgroup of initially HER2+ patients who had not been treated with trastuzumab. This supports the suggestion that HER2-directed therapy reduces the prognostic value of CTC enumeration.

Further, in the studies from Munzone et al. a high percentage (29 %) of samples contained 1–4 CTCs – a group which is classified as "CTC-negative" in the other studies [45]. Interestingly, this intermediate group has a much higher prognostic power in HER2- negative tumors – meaning that already one CTC makes the difference between good and worse prognosis. In this subgroup, this intermediate CTC-group has the same survival probability as patients with > 5 CTC/7.5 ml blood. In contrast, for patients with HER2-positive tumors the survival probability of patients with 1–4 CTC/7.5 ml blood tends to be the same as for CTC-negative patients. Hypothetically, different cut-offs might be required for different molecular subtypes of the disease. It would be interesting to know about heterogeneity and individual metastatic potential of each CTC in these groups. This observation emphasizes the need for a thorough and standardized analysis of the CTC images provided by CellSearch™ system.

Early breast cancer

Data on CTCs in different subtypes of non-metastatic breast cancer are inconsistent. The largest dataset including 2026 patients was published recently by Rack et al. [33] (Table 2). No association was found between CTC-positivity and luminal, basal-like, or HER2-positive tumors. Presence of CTC was a strong predictor for worse clinical outcome; however, when the authors refined their investigation by adding the information about the tumors' intrinsic subtype the CTCs' prognostic power was restricted to the largest subgroup of patients with luminal tumors.

Contrary to these findings are the results from the study by Hwang et al.; the authors retrospectively evaluated 166 patients with operable breast cancer (stage I–IIIA) which had not been pre-treated [52]. After surgery patients with HER2-positive tumors did not receive adjuvant treatment with trastuzumab. CK20 mRNA-positive blood was detected in 37 of 166 patients (22.3 %). These CK20 mRNA-positive patients had less favorable outcomes in terms of MFS and OS than patients with CK20 mRNA-negative patients. When the breast cancer samples were grouped into the molecular subtypes, luminal-A and luminal-B did not differ significantly in MFS and OS according to CTC-status. In contrast, patients with HER2-positive or TN disease and a CTC- positive status had shorter MFS and OS. Another study published in 2007 by Ignatiadis et al. analyzing the expression analysis of CK19 mRNA in 444 patients produced similar results to those by Hwang et al.: The presence of CK19 mRNA- positive CTCs was associated with shorter DFS and OS in the TN and HER2-positive subgroups but not in the ER+/HER2− subgroup [23, 53]. There is no plausible explanation for this inconsistency between these studies.

Table 2. CTCs and molecular subtypes in early breast cancer.

Author	n (CTC pos.)	Method	Frequency bias	All subtypes	Lum A	Lum B-like HER2−	Lum B-like HER2+	HER2+	TN
Rack (2014)	2026* (435)	CS	none	DFS, BCSS, OS	DFS, OS		n. s.		n. s.
Ignatiadis (2007)	444* (181) I–III	RT-PCR (CK19)	none	DFS, OS	n. s.	n. s.	n. d.	DFS, OS	DFS, OS
Hwang (2012)	166 (37) I–IIIA	RT-PCR (CK20)	none	MFS, OS	n. s.	n. s.	n. s.	MFS,OS	OS (MFS: p = 0.051)

* All patients had average-to-high risk breast cancer and received chemotherapy. n. s.: not significant; CS: CellSearch™; BCSS: breast cancer-specific survival; DFS: disease-free sur- vival; OS: overall survival; MFS: metastasis-free survival

Conclusion

In conclusion, the presented results strongly confirm the independent prognostic value of CTC enumeration in both early and metastatic breast cancer patients. CTC evaluation might provide further information that could be useful for individualizing breast cancer treatment. However, CTC positivity and clinical relevance of CTC detection vary between breast cancer subtypes. Interestingly, metastatic patients with HR+/HER2− tumors are most likely to present with CTCs in peripheral blood; the prognostic relevance of CTCs in this subtype seems to be the highest. In HER2-positive and triple-negative disease, data on prognostic significance of CTCs are inconclusive. Possibly, the role of CTC enumeration is strongly influenced by previous targeted treatment. From this point of view, current and future trials with therapies targeting specific CTC phenotypes and genotypes might have an impact on prognosis in patients with metastatic breast cancer; ongoing European and German clinical trials, such as TREAT CTC and DETECT III, may thus substantially improve our understanding of HER2-negative metastatic disease.

Conflict of Interest

None.

References

1 Weigelt B, Peterse JL, van't Veer LJ. Breast cancer metastasis: markers and models. Nat Rev Cancer 2005; 5: 591–602

2 Polzer B, Klein CA. Metastasis awakening: the challenges of targeting minimal residual cancer. Nat Med 2013; 19: 274–275

3 Stoecklein NH, Klein CA. Genetic disparity between primary tumours, disseminated tumour cells, and manifest metastasis. Int J Cancer 2010; 126: 589–598

4 Cummings MC, Simpson PT, Reid LE et al. Metastatic progression of breast cancer: insights from 50 years of autopsies. J Pathol 2014; 232: 23–31

5 Riethdorf S, Muller V, Zhang L et al. Detection and HER2 expression of circulating tumor cells: prospective monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. Clin Cancer Res 2010; 16: 2634–2645

6 Fehm T, Hoffmann O, Aktas B et al. Detection and characterization of circulating tumor cells in blood of primary breast cancer patients by RT-PCR and comparison to status of bone marrow disseminated cells. Breast Cancer Res 2009; 11: R59

7 Fehm T, Muller V, Aktas B et al. HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. Breast Cancer Res Treat 2010; 124: 403–412

8 Aurilio G, Disalvatore D, Pruneri G et al. A meta-analysis of oestrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 discordance between primary breast cancer and metastases. Eur J Cancer 2014; 50: 277–289

9 Schmidt M, Fasching PA, Beckmann MW et al. Biomarkers in breast cancer– an update. Geburtsh Frauenheilk 2012; 72: 819–832

10 Perou CM, Sorlie T, Eisen MB et al. Molecular portraits of human breast tumours. Nature 2000; 406: 747–752

11 Sorlie T, Perou CM, Tibshirani R et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001; 98: 10869–10874

12 Yu K, Lee CH, Tan PH et al. Conservation of breast cancer molecular subtypes and transcriptional patterns of tumor progression across distinct ethnic populations. Clin Cancer Res 2004; 10: 5508–5517

13 Kennecke H, Yerushalmi R, Woods R et al. Metastatic behavior of breast cancer subtypes. J Clin Oncol 2010; 28: 3271–3277

14 Sotiriou C, Pusztai L. Gene-expression signatures in breast cancer. N Engl J Med 2009; 360: 790–800

15 Cheang MC, Chia SK, Voduc D et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst 2009; 101: 736–750

16 Goldhirsch A, Wood WC, Coates AS et al. Strategies for subtypes dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 2011; 22: 1736–1747

17 Paget S. Distribution of secondary growths in cancer of the breast. Lancet 1889; 1: 571

18 Fehm T, Sagalowsky A, Clifford E et al. Cytogenetic evidence that circulating epithelial cells in patients with carcinoma are malignant. Clin Cancer Res 2002; 8: 2073–2084

19 Rack B, Andergassen U, Janni W et al. CTCs in primary breast cancer (I). Recent Results Cancer Res 2012; 195: 179–185

20 Kang Y, Pantel K. Tumor cell dissemination: emerging biological insights from animal models and cancer patients. Cancer Cell 2013; 23: 573–581

21 Banys M, Hartkopf AD, Krawczyk N et al. Dormancy in breast cancer. Breast Cancer (Dove Med Press) 2012; 4: 183–191

22 Krawczyk N, Hartkopf A, Banys M et al. Prognostic relevance of induced and spontaneous apoptosis of disseminated tumor cells in primary breast cancer patients. BMC Cancer 2014; 14: 394

23 Ignatiadis M, Xenidis N, Perraki M et al. Different prognostic value of cytokeratin-19 mRNA positive circulating tumor cells according to estrogen receptor and HER2 status in early-stage breast cancer. J Clin Oncol 2007; 25: 5194–5202

24 Zebisch M, Andergassen U, Kölbl AC et al. Nachweis zirkulierender Tumorzellen aus dem Blut von Brustkrebspatientinnen anhand der Genexpression von Cytokeratin 8, 18 und 19. Geburtsh Frauenheilk 2012; 72: 65

25 Riethdorf S, Fritsche H, Muller V et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. Clin Cancer Res 2007; 13: 920–928

26 Cristofanilli M, Budd GT, Ellis MJ et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004; 351: 781–791

27 Bidard FC, Peeters DJ, Fehm T et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol 2014; 15: 406–414

28 Hayes DF, Cristofanilli M, Budd GT et al. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res 2006; 12: 4218–4224

29 Bidard FC, Fehm T, Ignatiadis M et al. Clinical application of circulating tumor cells in breast cancer: overview of the current interventional trials. Cancer Metastasis Rev 2013; 32: 179–188

30 Smerage JB, Barlow WE, Hortobagyi GN et al. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J Clin Oncol 2014; 32: 3483–3489

31 Paoletti C, Muniz MC, Thomas DG et al. Development of circulating tumor cell-endocrine therapy index in patients with hormone receptor positive breast cancer. Clin Cancer Res 2014; DOI: 10.1158/1078-0432.CCR-14-1913

32 Wulfing P, Borchard J, Buerger H et al. HER2-positive circulating tumor cells indicate poor clinical outcome in stage I to III breast cancer patients. Clin Cancer Res 2006; 12: 1715–1720

33 Rack B, Schindlbeck C, Juckstock J et al. Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. J Natl Cancer Inst 2014; DOI: 10.1093/jnci/dju066

34 Slade MJ, Payne R, Riethdorf S et al. Comparison of bone marrow, disseminated tumour cells and blood-circulating tumour cells in breast cancer patients after primary treatment. Br J Cancer 2009; 100: 160–166

35 Pierga JY, Bidard FC, Mathiot C et al. Circulating tumor cell detection predicts early metastatic relapse after neoadjuvant chemotherapy in large operable and locally advanced breast cancer in a phase II randomized trial. Clin Cancer Res 2008; 14: 7004–7010

36 Lucci A, Hall CS, Lodhi AK et al. Circulating tumour cells in non-metastatic breast cancer: a prospective study. Lancet Oncol 2012; 13: 688– 695

37 Xenidis N, Ignatiadis M, Apostolaki S et al. Cytokeratin-19 mRNA-positive circulating tumor cells after adjuvant chemotherapy in patients with early breast cancer. J Clin Oncol 2009; 27: 2177–2184

38 Fehm T, Krawczyk N, Solomayer EF et al. ERalpha-status of disseminated tumour cells in bone marrow of primary breast cancer patients. Breast Cancer Res 2008; 10: R76

39 Kalinsky K, Mayer JA, Xu X et al. Correlation of hormone receptor status between circulating tumor cells, primary tumor, and metastasis in breast cancer patients. Clin Transl Oncol 2015; DOI: 10.1007/s12094-015-1275-1

40 Fehm T, Muller V, Alix-Panabieres C et al. Micrometastatic spread in breast cancer: detection, molecular characterization and clinical relevance. Breast Cancer Res 2008; 10 (Suppl. 1): S1

41 Giordano A, Giuliano M, De Laurentiis M et al. Circulating tumor cells in immunohistochemical subtypes of metastatic breast cancer: lack of prediction in HER2-positive disease treated with targeted therapy. Ann Oncol 2012; 23: 1144–1150

42 Bidard FC, Mathiot C, Degeorges A et al. Clinical value of circulating endothelial cells and circulating tumor cells in metastatic breast cancer patients treated first line with bevacizumab and chemotherapy. Ann Oncol 2010; 21: 1765–1771

43 Nole F, Munzone E, Zorzino L et al. Variation of circulating tumor cell levels during treatment of metastatic breast cancer: prognostic and therapeutic implications. Ann Oncol 2008; 19: 891–897

44 Pierga JY, Hajage D, Bachelot T et al. High independent prognostic and predictive value of circulating tumor cells compared with serum tumor markers in a large prospective trial in first-line chemotherapy for metastatic breast cancer patients. Ann Oncol 2012; 23: 618–624

45 Munzone E, Botteri E, Sandri MT et al. Prognostic value of circulating tumor cells according to immunohistochemically defined molecular sub- types in advanced breast cancer. Clin Breast Cancer 2012; 12: 340–346

46 Wallwiener M, Hartkopf AD, Baccelli I et al. The prognostic impact of circulating tumor cells in subtypes of metastatic breast cancer. Breast Cancer Res Treat 2013; 137: 503–510

47 Krawczyk N, Meier-Stiegen F, Banys M et al. Expression of stem cell and epithelial-mesenchymal transition markers in circulating tumor cells of breast cancer patients. Biomed Res Int 2014; 2014: 415721

48 Zeng Q, Li W, Lu D et al. CD146, an epithelial-mesenchymal transition inducer, is associated with triple-negative breast cancer. Proc Natl Acad Sci U S A 2012; 109: 1127–1132

49 Punnoose EA, Atwal SK, Spoerke JM et al. Molecular biomarker analyses using circulating tumor cells. PLoS One 2010; 5: e12517

50 Sieuwerts AM, Mostert B, Bolt-de Vries J et al. mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. Clin Cancer Res 2011; 17: 3600– 3618

51 Georgoulias V, Bozionelou V, Agelaki S et al. Trastuzumab decreases the incidence of clinical relapses in patients with early breast cancer presenting chemotherapy-resistant CK-19 mRNA-positive circulating tumor cells: results of a randomized phase II study. Ann Oncol 2012; 23: 1744–1750

52 Hwang SB, Bae JW, Lee HY et al. Circulating tumor cells detected by RT-PCR for CK-20 before surgery indicate worse prognostic impact in triple-negative and HER2 subtype breast cancer. J Breast Cancer 2012; 15: 34–42

53 Ignatiadis M, Perraki M, Apostolaki S et al. Molecular detection and prognostic value of circulating cytokeratin-19 messenger RNA-positive and HER2 messenger RNA-positive cells in the peripheral blood of women with early-stage breast cancer. Clin Breast Cancer 2007; 7: 883–889

4 Findings and future directions

The presence of CTCs in the peripheral blood is associated with impaired clinical outcome in both primary and metastatic breast cancer. Thus, intensive research on optimized CTC enrichment, detection and characterization is the key to understand the biology of CTCs and systemic cancer progression, and consequently might lead to the establishment of new treatment options for breast cancer patients. The present work was conceived to shed more light on the biology of CTCs with regard to an optimized “liquid biopsy” including efficient CTC capturing and their molecular analysis: two workflows were established which could be successfully implemented into patient sample analysis. One workflow allows for improved EpCAM-independent enrichment and further characterization of CTCs with a potential aggressive and metastases-inducing phenotype. The other workflow enables analysis of CTCs on the genomic level to determine a potential treatment resistance mechanism, i.e. activating *PIK3CA* hot spot mutations.

EpCAM-independent enrichment of CTCs

Findings: An immunomagnetic bead-based, highly flexible multi-marker assay enabling capturing of $EpCAM^{neg}$ CTCs from breast cancer blood samples was established. By targeting diverse cell surface antigens using antibodies against Trop2, CD49f, c-Met, CK8, CD44, ADAM8, CD146, and hyaluronic acid, 29 blood fractions depleted for EpCAM-positive cells from 25 patients were analyzed. In total, 95 potential $EpCAM^{neg}$ CTCs in 69% of the samples were identified. Additionally, 1069 $EpCAM^{neg}$ dual-positive cells ($CK^{pos}/CD45^{pos}$) in 28 out of 29 samples were detected. Using single-cell array-based comparative genomic hybridization (aCGH) the malignant nature of $EpCAM^{neg}$ CD44-enriched cells has successfully been proven. Similar chromosomal aberrations suggest a clonal relationship between $EpCAM^{pos}$ and $EpCAM^{neg}$ cells from the same patient, albeit disparate aberrant chromosomal patterns (on chromosomes 5 and 8p) were apparent as well.

Limitations: Cells that are missed by the EpCAM-positive selection through the CellSearch® are per definition designated as ‘EpCAM-negative’. Thus, EpCAM-depleted samples still might contain few $EpCAM^{pos}$ or $EpCAM^{low}$ cells. In addition, many different purification schemes, i.e. different antibody-bead combinations, have been applied as a proof of principle for this enrichment approach. Due to a high inter-patient variability and the unpredictability of epitope/antigen expression levels in different blood samples, the application of other markers/schemes might have attained completely different results. Furthermore, since a cytokeratin staining - as only accepted proof for a CTC - was used to

identify all recovered cells, CTCs with a fully developed mesenchymal phenotype might have been missed by this approach.

Future work: Whether cells omitted by EpCAM-based enrichment are indeed EpCAM-negative has yet to be provided, e.g. by immunofluorescence. While the clinical significance and utility of $EpCAM^{pos}$ CTCs is well-established, the relevance of $EpCAM^{neg}$ CTCs needs more comprehensive investigation, and this also applies to $EpCAM^{neg}$ $CK^{pos}/CD45^{pos}$ events. To identify distinct cell surface proteins that are associated with certain tumor characteristics the patient cohort has to be further expanded with respect to sample number and additional tumor subtypes. Moreover, the large variety of markers has to be narrowed to a minimum of comparable purification schemes, e.g. by applying bead preparations in a rather combined/mixed way, in order to finally determine an antibody cocktail which is evidently recovering cells of malignant origin. To this end, further genomic analyses, e.g. by aCGH and/or sequencing, of $EpCAM^{pos}$ and $EpCAM^{neg}$ CTC subpopulations are required to clarify clonal relationship of these cells, as well as to shed light onto inter- and intra-patient heterogeneity.

PIK3CA mutational analysis by SNaPshot

Findings: A methodology comprising CTC enrichment via CellSearch®, whole genome amplification (WGA) and the SNaPshot assay (PCR amplification & multiplex primer extension) was applied for the characterization of CTCs. The SNaPshot technology represents a simple and inexpensive tool which enables the simultaneous analysis of several single nucleotide polymorphisms (SNPs) within two exons of the *PIK3CA* gene in CTCs of metastatic breast cancer patients. Overall, 57 CTC-positive blood samples from 44 patients were investigated. Therein, twelve hotspot mutations in either *PIK3CA* exon 9/E545K (6/12, 50%) or exon 20/H1047R (6/12, 50%) in 15.8% of the patient samples were detected. Additionally, inter- and intra-patient heterogeneity could be observed. A 100% concordance between SNaPshot results and sequencing was achieved. Further, it has been demonstrated that the SNaPshot assay can detect *PIK3CA* mutations in single deposited and assayed breast cancer cells (MCF7, T47D).

Limitations: In general, the application of the SNaPshot assay is limited to a small number of known SNPs and does not allow the discovery of unknown mutations. Additionally, since a diploid human single cell contains only approx. 6 picograms genomic DNA, whole genomes were amplified prior to gene-specific PCR/SNaPshot: WGA might cause allelic and amplification bias, polymerase errors, and consequently false findings. Mutational analysis for patient samples was conducted with a pool of CTCs and not with single cells, potentially leading to a loss of information related to intra-patient heterogeneity. Besides

this, detection sensitivity of mutant alleles might be decreased due to contaminating leukocytes which are co-enriched with the CellSearch® system.

Future work: PIK3CA SNaPshot analysis may be further used to characterize e.g. single HER2-positive CTCs in patients that are resistant against HER2-targeted therapy regimens using trastuzumab or lapatinib. Since specific PI3K inhibitors are available, rapid mutational screening will be indispensable to stratify breast cancer patients for individual therapies. Besides analyzing *PIK3CA* hotspot mutations, the assay can be adopted easily for other therapeutically relevant targets such as ER, EGFR or PTEN, provided that hotspot mutations are known. However, genotyping of individual CTCs will also be accomplished by analyzing WGA products for a selected set of genes by panel sequencing (e.g. Ion AmpliSeq™ Cancer Hotspot Panel, Silicon Biosystems). Preliminary cooperation studies of our laboratory could demonstrate the feasibility of panel sequencing for whole genome amplified single cell templates including cell line and breast cancer CTCs.

Taken together, the herein presented work paves the way for a more thorough understanding of CTC biology. It enables the targeted single cell isolation of $EpCAM^{neg}$ CTCs as well as their molecular characterization on DNA/RNA and protein level. Thereby, it will help to feature answers to the urging question whether the presence of different CTC subpopulations is reflected in the clinical outcome of metastatic breast cancer patients.

5 Summary

Breast cancer is a systemic disease with primary tumors shedding tumor cells already at an early stage into the blood circulation. The circulating tumor cells (CTCs) represent the putative precursors of subsequent metastatic disease. A key to better understand systemic cancer progression and to improve the effectiveness of systemic therapy might lie in a holistic and comprehensive clarification of CTC biology. Thus, it is important to characterize CTCs at various molecular levels (DNA/RNA/protein), whereby at first their specific and efficient enrichment is needed. With regard to CTC enumeration, the EpCAM (Epithelial Cell Adhesion Molecule)-based CellSearch® system has facilitated CTC research enormously. During dissemination, however, CTCs might downregulate or completely lose their EpCAM expression triggered by a process called epithelial-mesenchymal-transition (EMT). Consequently, the question remains whether such non-epithelial-like CTCs are missed by an EpCAM-dependent CTC enrichment approach. Especially these cells are supposed to be more resistant against therapeutic agents and might represent the metastasis-relevant CTC population. In order to get access to this $EpCAM^{low/neg}$ CTC subset, a novel EpCAM-independent enrichment strategy analyzing blood from metastatic breast cancer patients was established. By immunomagnetic bead-based multi-marker targeting of proteins presented by tumor cells on their surface (e.g. Trop2, CD49f, c-Met, CK8, CD44, ADAM8, CD146) 29 blood samples depleted for $EpCAM^{pos}$ cells from 25 patients were examined. 95 potential $EpCAM^{neg}$ CTCs in 69% of the samples were identified. Further, $EpCAM^{neg}$ cytokeratin (CK)/CD45-double positive cells could be traced in 28 out of 29 samples. Genomic analysis confirmed the malignant origin of $EpCAM^{neg}$ cells captured with CD44 antibodies. In the future, further comparative analysis of $EpCAM^{neg}$ CTCs and their $EpCAM^{pos}$ counterparts will provide new insights into tumor cell heterogeneity.

Additionally, mutational analysis of CTCs might contribute to unravel intratumoral heterogeneity and to select for resistant tumor cells under biological therapies. To this end, simultaneous analysis for several activating single nucleotide polymorphisms within the *PIK3CA* (Phosphatidylinositol-3-kinase, catalytic subunit alpha) gene in $EpCAM^{pos}$ CTCs was conducted: 57 blood samples from 44 patients were investigated by a workflow comprising the SNaPshot technology. Thereby, 12 mutations in exon 9 (E545K, 50%) and exon 20 (H1047R, 50%) in 15.8% of the patient samples were identified. The detection of mutations in single cells was successful as well. Mutational analysis in CTCs can be used to study resistance to targeted therapies such as against HER2 or against other breast cancer relevant targets.

Taken together, the herein presented methods – suitable for investigating distinct $EpCAM^{pos}$ and $EpCAM^{neg}$ CTC subsets on the molecular level - represent valuable and promising tools for improved breast cancer diagnostics, monitoring of treatment efficacy and will pave the way for new findings in anti-cancer research.

6 Zusammenfassung

Brustkrebs ist eine systemische Erkrankung, bei der sich bereits in einem frühen Stadium Tumorzellen vom Primärtumor absiedeln und in den Blutkreislauf gelangen. Diese zirkulierenden Tumorzellen (CTCs) gelten als vermeintliche Vorläufer für eine nachfolgende Metastasierung. Der Schlüssel zu einem besseren Verständnis einer systemischen Tumorprogression sowie zur Optimierung der Therapiewirksamkeit könnte in einer ganzheitlichen und umfassenden Aufklärung der CTC-Biologie liegen. Daher ist es wichtig, CTCs auf verschiedenen molekularen Ebenen (DNA/RNA/Protein) zu charakterisieren, wobei zunächst eine spezifische und effiziente Anreicherung erforderlich ist. Im Hinblick auf die CTC-Anreicherung hat das EpCAM (*Epithelial Cell Adhesion Molecule*) basierte CellSearch®-System die CTC-Forschung enorm vorangetrieben. Während der Tumorzelldisseminierung können CTCs jedoch aufgrund der sogenannten Epithelial-mesenchymalen-Transition (EMT) die Expression von EpCAM herunterregulieren oder gar völlig verlieren. Daher bleiben CTCs mit geringer oder fehlender EpCAM Expression von EpCAM abhängigen Anreicherungssystemen möglicherweise unentdeckt. Gerade diese Zellen könnten jedoch gegenüber Therapeutika widerstandsfähiger und für die Metastasierung relevant sein. Um sich auch jene EpCAM-schwache/-negative Untergruppe der CTCs zugänglich zu machen, wurde eine neuartige, EpCAM unabhängige Anreicherungsstrategie entwickelt, mit der die Analyse von Blutproben aus Patientinnen mit metastasiertem Brustkrebs möglich ist. Mit Hilfe einer immunmagnetischen Bead-Anreicherung und Antikörpern gegen verschiedene Oberflächenmarker, z. B. gegen Trop2, CD49f, c-Met, CK8, CD44, ADAM8, CD146, wurden 29 Blutproben, bei denen die EpCAM-positiven Zellen zuvor entfernt wurden, von 25 Patientinnen untersucht. Dabei konnten 95 potenzielle CTCs in 69% der Proben identifiziert werden. In 28 der 29 Proben wurden darüber hinaus EpCAM-negative, Cytokeratin (CK)-/CD45 doppelt positive Zellen detektiert. Eine Genomanalyse lieferte den Nachweis über den malignen Ursprung von EpCAM-negativen Zellen, die mit CD44-spezifischen Antikörpern isoliert worden waren. In Zukunft wird man durch weitere vergleichende genomische Analysen der EpCAM-positiven und EpCAM-negativen CTCs neue Erkenntnisse über die Tumorzell-Heterogenität gewinnen können.

Auch der Nachweis von Mutationen in CTCs kann dazu beitragen, die zelluläre Heterogenität innerhalb eines Tumors zu enträtseln und solche CTCs aufzuspüren, die sich resistent gegenüber biologischen Therapieverfahren zeigen. Zu diesem Zweck wurden mehrere aktivierende Einzelnukleotid-Polymorphismen im *PIK3CA* (Phosphoinositid-3-Kinasen, alpha Untereinheit)-Gen simultan für EpCAM-positive CTCs analysiert. Dies

konnte mit einem Arbeitsablauf, der die SNaPshot-Technologie beinhaltet, realisiert werden: 57 Blutproben von 44 Patientinnen wurden untersucht, wobei 12 Mutationen in Exon 9 (E545K, 50%) und Exon 20 (H1047R, 50%) in 15,8% aller Proben identifiziert werden konnten. Der Mutationsnachweis in Einzelzellen war ebenfalls erfolgreich. Diese Mutationsanalysen in CTCs erlauben es künftig, Resistenzen gegenüber zielgerichteten Therapien, wie z. B. gegen HER2 oder andere für Brustkrebs relevante Targets, zu erforschen.

Zusammenfassend lässt sich festhalten, dass die hier dargestellten Methoden, die für die molekulare Untersuchung beider CTC-Untergruppen (EpCAM-positiv/-negativ) geeignet sind, wertvolle und vielversprechende Ansätze hinsichtlich der Brustkrebsdiagnostik sowie der Überwachung der Therapiewirksamkeit repräsentieren und neue Wege in der Krebsforschung ebnen werden.

Appendix

A *Statement*

Hiermit erkläre ich, Helen Schneck, dass ich die vorliegende Dissertation selbstständig verfasst und bei keiner anderen Universität bzw. Fakultät in der vorgelegten oder einer ähnlichen Form eingereicht habe. Für die Anfertigung der Dissertation habe ich keine anderen als die angegebenen Hilfsmittel verwendet. Die Stellen, die anderen Arbeiten dem Wortlaut oder dem Sinn nach entnommen sind, wurden unter Angabe der dazugehörigen Quelle kenntlich gemacht.

Düsseldorf, den 18. Januar 2016

Helen Schneck

B *Abbreviations*

°C	Degree Celsius
α, β	alpha, beta
µ	Mikro
aa	Amino acid
ab	Antibody
aCGH	Array comparative genomic hybridization
AJCC	American Joint Committee on Cancer
ALDH1	Aldehyde dehydrogenase 1
APC	Allophycocyanin
ASCO	American Society of Clinical Oncology
BMBF	Bundesministerium für Bildung und Forschung
BRCA1/2	Breast cancer 1/2, early onset
BSA	Bovine serum albumine
CAMs	Cell adhesion molecules
CC	CellCelector™
CD	Cluster of differentiation
CDH1/2	Cadherin 1/2
CK	Cytokeratin
Col	Collagen
CS	CellSearch®
CSF-1	Colony stimulating factor 1
CTC	Circulating tumor cell
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma *in situ*
DDFS	Distant disease-free survival
DNA	Deoxyribonucleic acid
DOI	Digital object identifier
DTC	Disseminated tumor cell
e.g.	*exempli gratia*
E-/N-cadherin	epithelial/neural cadherin
ECM	Extracellular matrix
EDC	Ethyl(dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EGF(R)	Epidermal growth factor (receptor)
EMT	Epithelial-mesenchymal transition

EpCAM	Epithelial Cell Adhesion Molecule
EpEX/ICD	EpCAM extracellular domain/ intracellular domain
ER	Estrogen receptor
et al.	*et alii*
FCS	Fetal calf serum
FDA	Food and Drug Administration
FGF(R)R	Fibroblast growth factor (receptor)
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FN1	Fibronectin-1
g	Gram
HA	Hyaluronic acid
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
i.e.	*id est*
ICC	Immunocytochemistry
IDC	Invasive ductal carcinoma
IgG	Immunoglobulin G
ILC	Invasive lobular carcinoma
kDa	Kilo dalton
l	Liter
Lam	Laminin
m	Meter
M	Molar
MAGE-A3	Melanoma-associated antigen 3
MET	Mesenchymal–epithelial transition
MMP	Matrix metalloproteinase
MUC1	Mucin 1, cell surface associated
n	Nano
neg/pos	Negative/positive
NHS	*N*-hydroxysuccinimide
PAI1	Plasminogen activator inhibitor-1
PARP	Poly (ADP-ribose) polymerase
PBGD	Porphobilinogen deaminase
PBS	Phosphate buffered saline
PCR	Polymerase chain reactions
PE	Phycoerythrin

PFS	Progression-free survival
PI3K	Phosphatidylinositol-3-kinase
PIK3CA	Phosphatidylinositol-3-kinase, catalytic subunit alpha
PR	Progesterone receptor
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
TGF-ß	Transforming growth factor beta
TNF	Tumor necrosis factor
TNM	Tumor, node, metastasis
TRITC	Tetramethylrhodamine
US	United States
VEGF	Vascular endothelial growth factor
vs	*versus*
WGA	Whole genome amplification
ZEB1/2	Zinc finger E-box-binding homeobox 1/2

C *List of figures and tables*

FIGURES

TABLES

D *Wissenschaftlicher Lebenslauf*

Ausbildung

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